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Review

Microbial nitrogen transformations tracked by natural abundance isotope studies and microbiological methods: A review

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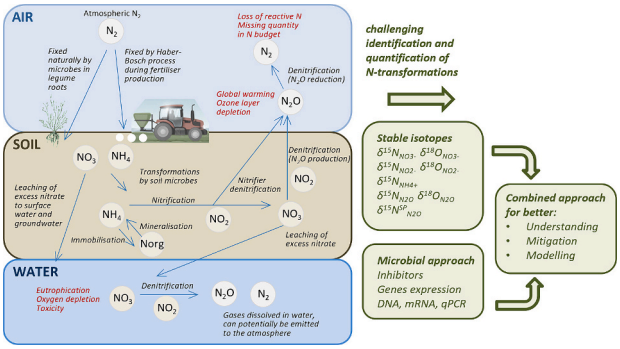
HIGHLIGHTS

- Promising combination of natural isotope and microbial approaches for tracking N cycling
- Detailed summary of N and O isotope effects for NO_3^-, NO_2^-, NH_4^+ and N_2O transformations
- Overview of microbes, enzymes and encoding genes involved in N transformations
- Examples of combined studies and a discussion about their possible application

GRAPHICAL ABSTRACT



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ABSTRACT

Nitrogen is an essential nutrient in the environment that exists in multiple oxidation states in nature. Numerous microbial processes are involved in its transformation. Knowledge about very complex N cycling has been growing rapidly in recent years, with new information about associated isotope effects and about the microbes involved in particular processes. Furthermore, molecular methods that are able to detect and quantify particular processes are being developed, applied and combined with other analytical approaches, which opens up new opportunities to enhance understanding of nitrogen transformation pathways. This review presents a summary of the microbial nitrogen transformation, including the respective isotope effects of nitrogen and oxygen on different nitrogen-bearing compounds (including nitrates, nitrites, ammonia and nitrous oxide), and the microbiological characteristics of these processes. It is supplemented by an overview of molecular methods applied for detecting and quantifying the activity of particular enzymes involved in N transformation pathways. This summary should help in the planning and interpretation of complex research studies applying isotope analyses of different N compounds and combining microbiological and isotopic methods in tracking complex N cycling, and in the integration of these results in modelling approaches.

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1. Introduction

Nitrogen (N) is an essential nutrient in the environment and a basic element in living organisms. It is one of the main chemical components in nucleic acids and proteins, and it exists in multiple oxidation states in nature. Atmospheric N_2 is the largest N reservoir and it is biologically accessible to nitrogen-fixing bacteria and archaea. N fixation and other transformations are mainly catalysed by microorganisms whose microbial activity influences N transformation processes and rates (Grzyb et al., 2021). Bio-available N is in deficit in natural environments and is mainly dependent on microbial reactions that change N oxidation states into usable N forms (Fig. 1).

Human activity affects N bioavailability, mainly due to agricultural practices associated with increased food production, such as the intensive application of synthetic fertiliser, which primarily contains reactive N in the forms of nitrate or ammonia, to maximise crop yields. Excess application of reactive N forms has caused a disturbance in the global N cycle, leading to greater N losses from soil (Haslun et al., 2018). Nitrogen is lost due to leaching, primarily as nitrate (NO_3^-) or ammonium (NH_4^+), to groundwater and surface water, and emission of gaseous forms of nitrogen oxides, e.g. nitric oxide (NO), nitrous oxide (N_2O) and nitrogen dioxide (NO_2), all of which is of environmental concern (Bijay-Singh and Craswell, 2021; Böhlke et al., 2006).

Excess nitrogen input into the environment is associated with changes in N cycling and balance, which results in serious environmental problems both for waters and the atmosphere. A high NO_3^- concentration in water may cause eutrophication and deterioration in water quality, and may pose a threat to human health (Rütting et al., 2018). Highly NH_4^+-contaminated groundwater is undrinkable due to its bad taste and odour, and may be associated with pathogenic contamination (Maharjan et al., 2020). N_2O is a potent greenhouse gas and an ozone layer-depleting gas (Butterbach-Bahl et al., 2013). Due to these serious threats, N input and its further fate in the environment must be well controlled and trackable. Understanding the link between N cycling processes and microbial community dynamics can provide insights that elucidate the sources and natural attenuation of mineral N in the environment. A major concern in the agricultural system is to develop strategies that improve soil fertility, increase N-use efficiency and avoid N accumulation or losses in the ecosystem. Furthermore, it is crucial to differentiate microbial pathways in complex biogeochemical N-cycling processes for better quantification of NH_4^+ and NO_3^- losses, NO_3^- production and N_2O emissions. It is essential to track and manipulate these processes, and consequently mitigate the negative environmental impacts associated with N losses (Fig. 1).

In recent years, studies on the naturally occurring N isotopes, ^{15}N and ^{14}N, and their isotope fractionation effects associated with microbial N transformation have contributed hugely to understanding of the N cycle (Denk et al., 2017, 2019). Stable isotope studies are applied to assess the various sources and sinks of different molecules in the N-cycling process. Variations in $^{15}N/^{14}N$ in NH_4^+, NO_2^- and NO_3^-, often complemented with variations in $^{18}O/^{16}O$ in NO_2^- and NO_3, can be used to quantify the associated fluxes during the production or consumption of these molecules (Magyar et al., 2021). Furthermore, stable isotope analyses of gaseous N_2O losses play a crucial role in identifying the sources and transformation pathways of N_2O and N_2 emissions, thanks to analyses of N_2O isotopocules (Yu et al., 2020b). Namely, for the linear N_2O molecule, in addition to the N and O isotopic signatures ($\delta^{15}N$ and $\delta^{18}O$), intramolecular ^{15}N distribution, known as *site preference* ($\delta^{15}N^{SP}$), can also be determined. This provides important additional information in the identification of various processes of N_2O production and

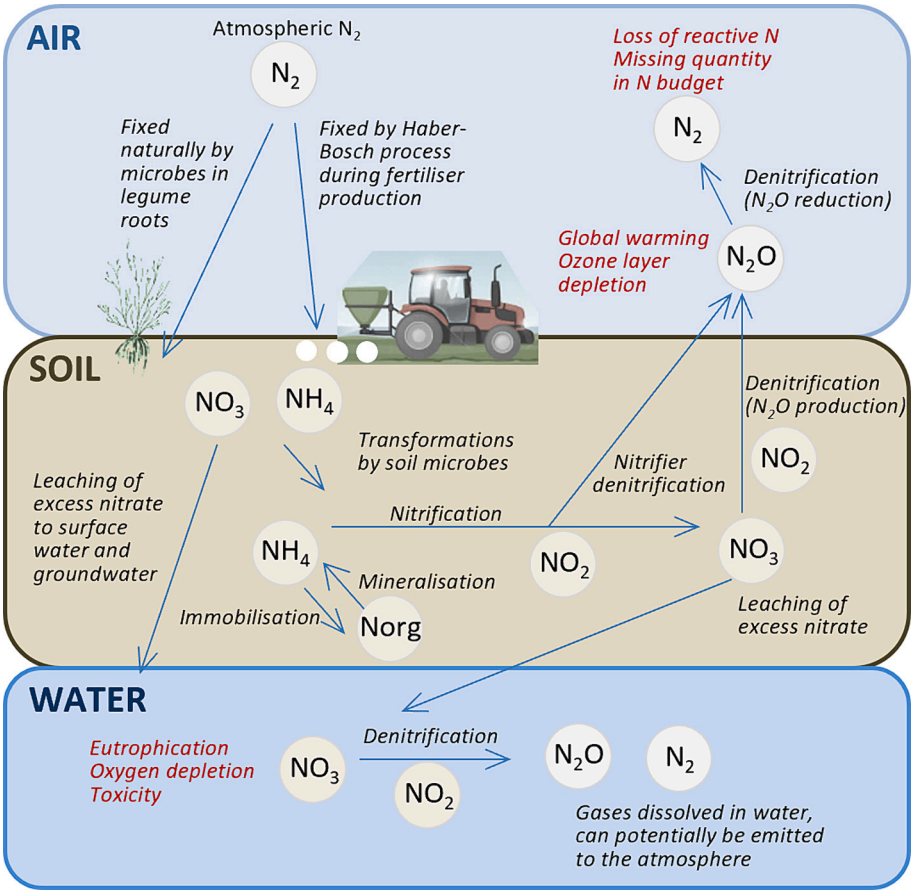

Fig. 1. Nitrogen sources and the main transformation pathways in the environment (described in black font) with their associated environmental problems (described in red font). N_2: nitrogen, NO_2: nitrogen dioxide, N_2O: nitrous oxide, NO_3: nitrate, NH_4: ammonium, N_{org}: organic nitrogen.

reduction (Toyoda et al., 2017; Yu et al., 2020b). The isotopic results are rich in information, but their interpretation is often challenging due to different patterns of isotopic fractionation for each of the analysed compounds in the N cycle. Hence, this review describes the isotope effects of different microbial N transformations for each of the N-cycle compounds that can be characterised isotopically. A combination of the isotope characteristics at different stages of the N cycle can be a powerful tool in unravelling the processes that take place, hence complex stable isotope datasets can help with the identification of sources of N pollution in the environment (Fig. 1).

However, isotopic analyses have several limitations. To make them useful for identifying particular pathways, it is necessary to have basic information about the isotope effects associated with particular N transformations. This knowledge mostly originates from pure culture studies in which particular microbial transformations are isolated and products/substrates are isotopically characterised (e.g. Ostrom et al., 2010; Rohe et al., 2017; Sutka et al., 2006). Although particular processes show characteristic isotope effects, the possible ranges for these values are quite large and may depend on the process rate, the microbes involved, e.g. bacteria, archaea or fungi, and the substrates used, e.g. molecular oxygen or water as an O source, etc. Furthermore, the ranges of characteristic isotope effects for different processes may overlap, preventing them from being distinguished (Denk et al., 2017). Since nitrogen cycling is very complex and multiple processes occur simultaneously (Fig. 2), it is often challenging to identify correctly the isotope mixing of different sources and isotope fractionation due to partial consumption of a particular compound (Lewicka-Szczebak et al., 2020, 2021). Production and consumption often occur simultaneously, and complex mathematical models are needed to quantify their balance properly (Lewicki et al., 2022). This review shows for each compound what the characteristic isotope values are for production and consumption due to a particular process, which is fundamental knowledge when including isotopic studies in modelling tasks.

Due to numerous co-existing processes and possibly overlapping ranges of characteristic isotopic signatures, parallel methods for process identification are often needed. Microbial approaches can serve well as complementary methods for the correct identification of N transformations. Microorganisms play important roles in nitrogen cycling as nitrogen-fixing bacteria and archaea, anammox bacteria, heterotrophic nitrifying microorganisms, and denitrifying bacteria, archaea and fungi. The ability to conduct a particular transformation, determined in the microbes' genetic information, can be detected by functional characterisation of the genes encoding particular enzymes involved in the N metabolism (Levy-Booth et al., 2014). Sophisticated microbiological methods are able to determine the gene expression and identify which microbiological processes can potentially occur (DNA-based studies) and which of them are currently active (RNA-based studies). When applying special methods for quantitative determination of gene transcription, it is even possible to determine the proportion of particular pathways. This allows selection of the processes that should be taken into account for a particular study, thereby minimising the often large uncertainty involved in predefining possibly co-existing pathways. The quantitative information on gene transcription can also be applied to cross-check and validate the results of isotope studies.

This review describes the different N-cycle processes and summarises the isotope fractionation effects associated with microbiologically-mediated N transformations. It also summarises the characteristic N and O isotopic signatures for various ecosystem processes and shows the possible linkage of these isotopic studies with microbiological methods. It builds on previous reviews of N isotope effects (Denk et al., 2017), N and O isotope characteristics of groundwater (Nikolenko et al., 2018) and N_2O isotope dynamics (Yu et al., 2020b), and includes numerous new isotope effects that have only recently been published, e.g. regarding heterotrophic nitrification and anammox processes. It also summarises the isotope effects governing the isotopic signature of NO_2^-, as a crucial, often overlooked intermediate in the N cycle (Lewicka-Szczebak et al., 2021). The focus here is on the linkage between isotope and microbial studies, which recent research shows to be highly promising (Li et al., 2022b; Masta et al., 2023).

2. Basics of stable isotope studies

N has two naturally occurring stable isotopes: ^{14}N (99.635 %) and ^{15}N (0.365 %). O has three stable isotopes: ^{16}O (99.757 %), ^{17}O (0.038 %) and ^{18}O (0.205 %). The stable isotope composition is expressed in delta (δ) units and per mil (‰) notation, defined in relation to international standards as follows:

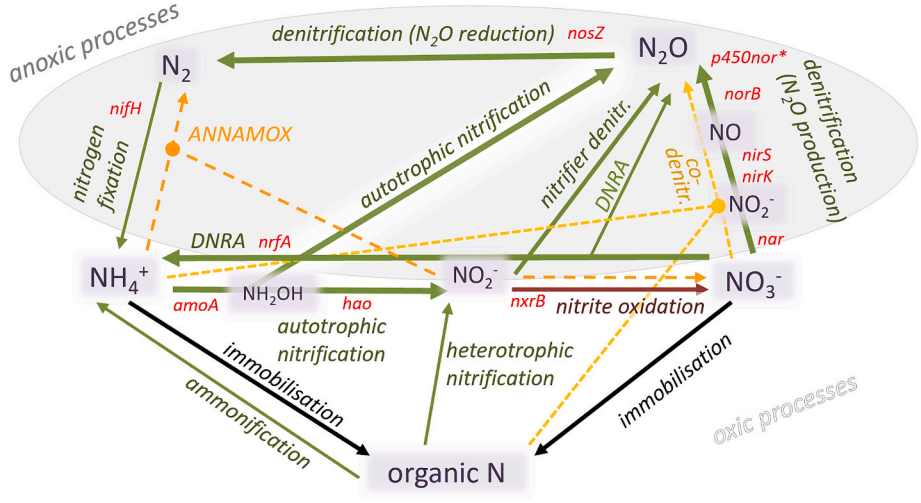

Fig. 2. Microbiological N transformation processes in the N cycle. The grey area indicates the processes occurring preferentially in anoxic conditions, and the white area represents the processes occurring in oxic conditions. Green arrows indicate processes associated with normal isotope effects (product depletion in heavy isotope), with the line's thickness correlated with the mean magnitude of the isotope effect for a particular process (data summarised in Table 2). The red arrow indicates the process characterised with an inverse isotope effect (product enrichment in heavy isotopes). Complex processes (of multiple substrates) with very limited knowledge on the associated isotope effects are marked with orange and yellow arrows (same type for the same processes, anammox and co-denitrification, respectively). Black arrows indicate processes of negligible isotope effects. Red italic font indicates the diagnostic genes encoding the main N transformations (* gene typical for fungi only).

$$\delta = R_{sample}/R_{standard} - 1 \quad (1)$$

where R_{sample}, $R_{standard}$ is the ratio of heavy isotope to light isotope in the analysed sample and the international standard, respectively. The positive and negative δ value represents respectively the enrichment and depletion in heavy isotopes in the analysed sample compared with the international standard (Sharp, 2007).

Isotope fractionation is a process that results in changes in the isotopic signatures of a product compared with a substrate. The magnitude of this change is described as the isotope enrichment factor ($\varepsilon_{p/s}$), also known as the isotope effect (Sharp, 2007):

$$\varepsilon_{p/s} = R_p/R_s - 1 \approx \delta_p - \delta_s \quad (2)$$

where R_p and R_s are the isotope ratio of the product and initial substrate, respectively. The $\varepsilon_{p/s}$ value represents the approximate difference in the isotopic signature between the product and substrate. Note that the definition of ε values may differ in various publications and can be expressed as the product/substrate or substrate/product ratio. Here the isotope effect is always expressed as $\varepsilon_{p/s}$, as defined above, which means that negative ε values indicate a normal isotope effect, i.e. the depletion of the product in heavy isotopes. Larger isotope effects, i.e. greater differences between the product and substrate isotope signatures, are denoted with more negative ε values and smaller isotope effects with ε values closer to 0. Positive $\varepsilon_{p/s}$ values indicate the inverse isotope effect, i.e. enrichment of the product in heavy isotopes.

Importantly, isotope fractionation also results in changes in the isotopic composition of the substrate, depending on the progress of its consumption. Hence, even when only the isotope signature of the substrate (initial and residual) is analysed, isotope fractionation can be defined based on changes in the substrate pool, comparing its final (δ_S) and initial (δ_{S0}) isotopic signatures and taking into account the residual substrate fraction (*f*) in an open (Eq. (3)) or closed system approach (Eq. (4)) (Denk et al., 2017):

$$\varepsilon_{S/S0} \approx (\delta_S - \delta_{S0})/ln(f) \quad (3)$$

$$\varepsilon_{S/S0} \approx (\delta_S - \delta_{S0})/(f - 1) \quad (4)$$

With this definition, the negative ε values also indicate the enrichment of the residual substrate in heavy isotopes compared with the initial substrate, similarly as in Eq. (2).

In this review, if originally published in a different manner, all ε values have been transformed and expressed in accordance with Eqs. (2)–(4). Nitrogen and oxygen isotope effects are denoted as $^{15}\varepsilon$ and $^{18}\varepsilon$, respectively.

For N_2O, site preference values ($\delta^{15}N^{SP}$) are also defined, representing the difference in $\delta^{15}N$ value between the central ($\delta^{15}N^{\alpha}$) and peripheral N position ($\delta^{15}N^{\beta}$) in the linear N_2O molecule (Toyoda and Yoshida, 1999):

$$\delta^{15}N^{SP} = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$$

3. Basics of complementary microbiological methods

3.1. Gene expression

Molecular-based analyses of selective enzymes provide precise tools for identifying particular N transformation pathways (Butterbach-Bahl et al., 2013; Stein and Yung, 2003). In approaches based on DNA abundance measurements, polymerase chain reaction (PCR) is applicable for identifying genetic potential by analysing functional genes encoding the specific enzymes responsible for a particular reaction step in pure culture or ecosystem studies. However, information on the actual activity of a particular process is still difficult to capture and requires an analysis of gene expression by examining cDNA reverse-transcribed from mRNA. If gene expression is analysed with real-time PCR, specifically the quantitative PCR method (qPCR), it can be used to assess the activity and even relative magnitude of this process (Kralik and Ricchi, 2017; Rohe et al., 2020). However, RNA-based methods can currently be performed well in pure culture studies, but have their limitations in terms of sample size in natural systems such as soil or water bodies (Espenberg et al., 2018).

To be able to apply this analytical approach of molecular studies in practice, predefined functional marker genes encoding the key enzymes of particular processes are required. Numerous studies have developed gene-specific primers for N transformations that are able to detect and quantify the genes encoding particular enzymes, and consequently allow the identification of the microbial structure and active pathways. A summary of these studies is given in Table 1.

For nitrifying communities gene primers are applied to identify ammonia-oxidising bacteria and archaea with *amoA* genes (Brochier-Armanet et al., 2008; Purkhold et al., 2000) and nitrite-oxidising bacteria with *nrxB* genes (Pester et al., 2014). However, the occurrence of *amoA* genes has also recently been identified in a group of bacteria within the *Nitrospira* genus, known as "comammox" *Nitrospira*, which are capable of performing complete oxidation of NH_4 to NO_3 (Pjevac et al., 2017). This study introduced new primer sets designed for the precise detection of the *amoA* gene in comammox *Nitrospira*. These primers offer high specificity and broad applicability for various PCR-based techniques, suggesting the presence of comammox *Nitrospira* in different environmental samples, with *amoA* gene abundance comparable to that of other ammonia oxidisers.

For denitrifying communities there are numerous primers available. The genes encoding nitrite reductase, which is the key enzyme in the dissimilatory denitrification process, are especially useful. The reduction of NO_2^- to NO can be catalysed by the enzymes encoded by two different nitrite reductase genes: *nirK* containing Cu and *nirS* containing cytochrome cd_1. Both types are structurally different but functionally equivalent, and have been found in different strains of the same species. *nirS* is more widely distributed, while *nirK* is found in only 30 % of denitrifiers but in a wider range of physiological groups (Braker et al., 1998). Consequently, not all organisms capable of reducing NO_2^- can also

Table 1
Summary of known genes encoding particular enzymes in major N transformation pathways.

Gene	Encodes	Marker of	Reference
amoA	Ammonia monooxygenase	Ammonia-oxidising bacteria (AOB), ammonia-oxidising archaea (AOA) comammox	(Brochier-Armanet et al., 2008; Pjevac et al., 2017; Purkhold et al., 2000)
nxrB	Nitrite oxidoreductase	Nitrite-oxidising bacteria (NOB)	(Pester et al., 2014)
hao	Hydroxylamine oxidoreductase	Ammonia-oxidising bacteria (AOB)	(Schmid et al., 2008)
nirK	NO_2 reductase containing Cu	Denitrifying bacteria	(Braker et al., 1998)
nirS	NO_2 reductase containing cytochrome *cd*1	Denitrifying bacteria	(Braker et al., 1998)
norB	NO reductase	Denitrifying bacteria	(Braker and Tiedje, 2003)
qNorB	Quinol-oxidising NO reductase	Denitrifying bacteria and non-denitrifying strains	(Braker and Tiedje, 2003)
cNorB	Cytochrome c oxidising NO reductase	Denitrifying bacteria	(Braker and Tiedje, 2003)
p450nor	NO reductase	Denitrifying fungi	(Higgins et al., 2016; Rohe et al., 2020)
nosZ	N_2O reductase	Denitrifying bacteria	(Scala and Kerkhof, 1998)
nrfA	Cytochrome c nitrite reductase	Dissimilatory nitrate reduction to ammonium	(Giacomucci et al., 2012)

reduce NO. Hence, the critical step for quantifying N_2O production from denitrification is the NO reduction to N_2O catalysed by NO reductase encoded with the *norB* genes (Braker and Tiedje, 2003).

N_2O reduction, as the final step in the denitrification pathway, is indicative of bacterial and archaeal denitrification, therefore the N_2O reductase (*nosZ*) gene is mostly unique to denitrifying bacteria, although a few non-denitrifier species capable of reducing N_2O have been identified (Scala and Kerkhof, 1998). Importantly, the *nosZ* genes include two distinct groups: the well-studied Clade I typical for denitrifiers (*nosZ-I*), and the novel Clade II found in diverse groups of microorganisms, most of which are non-denitrifiers (*nosZ-II)* (Shan et al., 2021). *nosZ-II* may play an important role in controlling the consumption of N_2O produced by processes other than denitrification (Sanford et al., 2012; Shan et al., 2021). Wide distribution of *nosZ* genes spanning 16 taxonomic groups indicates the capability of reducing N_2O to N_2 by diverse microorganisms, including bacteria and archaea (Sanford et al., 2012).

Higgins et al. (2016) explored fungal denitrifiers using molecular approaches targeting the *p450nor* gene, which is critical for N_2O production in fungi. The newly designed *p450nor*-targeted primers effectively identified fungal N_2O production potential in both denitrifying isolates and soil DNA samples. The primers were shown to complement existing sets, aiding in the assessment of fungal capacity to reduce NO_2^- to N_2O. Further, Rohe et al. (2020) used this molecular marker for RNA studies with pure fungal cultures of *Fusarium* sp. to target the fungal *p450nor* gene encoding NO reductase associated with fungal denitrification. Specific PCR and qPCR primers targeting *p450nor* were developed which amplified pure cultures of *Fusarium* sp., but failed in the case of *Chaetomium funicola*, which confirms a lack of N_2O production due to the absence of *p450nor* genes. Another popular marker for fungal denitrifiers is *fungal nirK,* but some experimental results indicate issues with its specificity against bacterial *nirK* (Ma et al., 2019).

3.2. Inhibition methods

Methods frequently used for separating out individual processes from the complex N cycle are based on applying inhibitors that are able to block particular reactions or groups of microorganisms selectively. These methods are used to detect, describe or quantify particular processes (Herrmann et al., 2007; Lin et al., 2021; Rohe et al., 2021) and manipulate the N cycle, e.g. with the aim of minimising losses of reactive N from agricultural areas (Coskun et al., 2017).

Nitrification inhibitors are applied to agricultural soils to mitigate nitrification and consequently increase N-use efficiency. This can be achieved by synthetic nitrification inhibitors and recently also by biological nitrification inhibitors, i.e. compounds naturally produced by plant roots (Akiyama et al., 2009; Coskun et al., 2017). A few studies have shown a successful reduction of soil NO_3^- (Cui et al., 2022) and soil N_2O emissions after application of nitrification inhibitors (Dittert et al., 2001; Wu et al., 2017). Applied inhibition techniques affect ammonia monooxygenase (AMO) and hydroxylamine dehydrogenase (HAO), which are responsible for catalysing NH_3 oxidation to NO_2^- and NO by reducing the enzyme's activity and influencing nitrification rates, both for ammonium-oxidising bacteria (AOB) and archaea (AOA) (Nardi et al., 2020). This can be achieved by competitive inhibition (compounds compete for AMO's active site), non-competitive inhibition (inhibitors cause conformational changes, e.g. allylthiourea, guanidine) and irreversible inhibition (inhibitors form stable covalent bonds with AMO's active site, e.g. acetylene and allyl disulfide) (Nardi et al., 2020).

Acetylene is one of the most widely used irreversible inhibitors to block nitrification (applied at a lower concentration of 0.1 kPa in the headspace) or block N_2O reduction to N_2 (at higher concentrations of 5-10 kPa in the headspace) (Nadeem et al., 2013; Well and Flessa, 2009; Wrage-Mönnig et al., 2018). Herrmann et al. (2007) applied acetylene to inhibit rapid NH_4^+ nitrification in the experiments, which improved the determination and control of N transformation processes. Wrage et al. (2004) used the acetylene inhibition to identify and quantify nitrifier denitrification in soils, however it was found that not all ammonia oxidisers can be inhibited successfully with this treatment. Numerous studies have applied acetylene to block N_2O reduction to N_2. In this application, N_2O is the final denitrification product, and hence represents the total denitrification flux (Felber et al., 2012; Lin et al., 2021). Moreover, with the inhibition of N_2O consumption, it is possible to determine the isotopic signatures of the N_2O originally produced that is unaffected by the reduction processes (Lewicka-Szczebak et al., 2014, 2017; Well and Flessa, 2009). This results in the elimination of additional isotopic fractionation associated with N_2O reduction, and hence allows more precise determination of the isotope characteristics of particular N_2O sources (Lewicka-Szczebak et al., 2017; Rohe et al., 2021; Yu et al., 2020b). However, the application of acetylene methods requires special conditions, i.e. a short experimental time and an anoxic atmosphere (Nadeem et al., 2013), therefore only N_2O originating from bacterial denitrification can actually be determined using this approach, since nitrification processes are inhibited and fungal denitrification does not take place in strict anaerobic conditions (Lewicka-Szczebak et al., 2017). Moreover, this method is one of the possible approaches for estimating the progress of N_2O consumption, and can theoretically be applied to determine the isotope effect associated with N_2O reduction to N_2 by comparing the acetylated and non-acetylated treatments (Lewicka-Szczebak et al., 2014). However, some studies have shown difficulties with achieving complete inhibition of N_2O reduction, and consequently with correctly determining the N_2O reduction isotope effects and total denitrification losses of $N_2 + N_2O$ (Felber et al., 2012; Yu et al., 2020b). Since the completeness of N_2O reduction inhibition may vary with the experimental setup, soil type, moisture etc., it is advisable to undertake this approach with an independent control of complete N_2O inhibition, e.g. with $^{15}N_2$ measurements in a ^{15}N tracing approach (Lewicka-Szczebak et al., 2017).

Selective inhibition of microbial groups can be applied to inhibit the growth of particular microorganisms, e.g. fungi or bacteria. This method can theoretically be helpful in distinguishing between pathways mediated by different microorganisms, e.g. fungal and bacterial denitrification processes. Anderson and Domsch (1975) developed the substrate-induced respiration with selective inhibition (SIRIN) method aimed at distinguishing between bacterial and fungal contributions to CO_2 respiration. The modified SIRIN approach with bactericide streptomycin and fungicide cycloheximide has frequently been used for source partition of N_2O production (Blagodatskaya et al., 2010; Crenshaw et al., 2008; Long et al., 2013). However, it should be noted that this approach only inhibits organisms in the growth phase, and hence it is necessary to check the production background in the treatment with all the inhibitors applied to obtain information about non-inhibited organisms. This background production may be very significant and may consequently lead to this approach being unsuccessful at quantifying N_2O production from different pathways and determining these pathways' isotope characteristics (Rohe et al., 2021). Other substances have also been tested and applied, such as the bactericide bronopol and the fungicide captan (Ladan and Jacinthe, 2016). However, use of the improved inhibition approach still needs cross-validation with other independent methods.

Selective inhibition techniques can be also applied to determine the role of bacteria and archaea by distinguishing between AOA, AOB and comammox. Hydrazine and phenylhydrazine can disrupt both ammonia and hydroxylamine oxidation in AOA. The inhibitory effects of hydrazines are similar for AOA and AOB (Schatteman et al., 2022). The role of comammox has been determined through laboratory microcosm experiments with nitrification inhibitors: 2-Octyne (C_8H_{14}) specifically targeted AOB, while acetylene (C_2H_2) inhibited both AOA and AOB, as well as comammox *Nitrospira*. The findings suggest that comammox *Nitrospira* may have a notable impact on the soil nitrogen cycle (Li et al., 2019).

4. Main N-bearing compounds of the N cycle

4.1. Ammonium

Ammonium (NH_4^+), one of the major forms of reactive N in the environment, is primarily produced by mineralisation of organic N forms. NH_4^+ biodegradation occurs during nitrification, nitrifier denitrification and anammox processes through several microbial reactions and transformation pathways. However, excess NH_4^+ in the system, especially in groundwater, a major drinking water source, may reduce the effectiveness of chlorine disinfection and increase pathogenic contamination (Maharjan et al., 2020). Furthermore, in biologically-treated wastewater systems at high concentrations of NH_4^+ (above 60 $mg{\cdot}dm^{-3}$), the inhibition of nitrification processes has been observed (Paśmionka et al., 2021), hence it is essential to regulate NH_4^+ concentrations in groundwater. The $\delta^{15}N$ value of NH_4^+ ($\delta^{15}N_{NH4+}$) analyses can help with the identification of N sources and transformation processes and the attenuation of NH_4^+ in the environment.

Major anthropogenic sources of NH_4^+ in agricultural areas include application of synthetic fertilisers and manure. $\delta^{15}N_{NH4+}$ of fertilisers usually ranges between −7.4 ‰ and +5.1 ‰ (Kendall, 1998; Nikolenko et al., 2018; Vitòria et al., 2004; Wassenaar, 1995). Furthermore, sewage effluent and wastewater from treatment plants are a major source of NH_4^+ contamination in groundwater, varying between +5 ‰ and +9 ‰ (Cole et al., 2006; Liu et al., 2006; Robertson et al., 2012). Rainwater can be another source of NH_4^+, adding negative $\delta^{15}N_{NH4+}$ values that vary from −13.4 ‰ to +2.3 ‰ (Li et al., 2007). Decomposition of organic matter adds slightly depleted NH_4 of $\delta^{15}N_{NH4+}$ from −4 ‰ to −2 ‰ (Möbius, 2013) (Table 2, Fig. 3). In soil and water NH_4^+ undergoes microbial transformations that can significantly alter its isotopic signature (see Section 5).

4.2. Nitrate

NO_3^- is the dominant N species in groundwater and may originate from soil organic N oxidation, synthetic fertiliser, livestock waste, sewage effluent and atmospheric precipitation. Stable isotopes of N and O in NO_3^- ($\delta^{15}N_{NO3-}$ and $\delta^{18}O_{NO3-}$) have been reported to identify sources of NO_3^- contamination due to distinct isotopic values from source to source (Jung et al., 2020), characterise NO_3^- production by different pathways and quantify the amount of NO_3^- consumed due to denitrification (Bouskill et al., 2019; Sbarbati et al., 2018).

The $\delta^{15}N_{NO3-}$ value from precipitation is typically low (values ranging from −10 ‰ to +9 ‰) (Sharp, 2007), while in synthetic fertilisers, produced by atmospheric N fixation, $\delta^{15}N_{NO3-}$ values show quite a narrow range around 0 ± 3 ‰ (Kendall, 1998; Xue et al., 2009). Compared with inorganic fertilisers, $\delta^{15}N_{NO3-}$ is usually higher in manure and sewage, with values ranging from +3 ‰ to +35 ‰ (Bateman et al., 2005; Lorenzo et al., 2012; Vitòria et al., 2004; Widory et al., 2005; Xue et al., 2009). $\delta^{15}N_{NO3-}$ derived from the bacterial decomposition of organic matter from plants and animals ranges from +3 ‰ to +8 ‰ (Kendall and Aravena, 2000).

For more precise identification, major sources of NO_3^- are determined by studying dual stable isotopes $\delta^{15}N$ and $\delta^{18}O$. While $\delta^{15}N_{NO3-}$ for precipitation is low, the $\delta^{18}O_{NO3-}$ value of precipitation is high at between +20 ‰ and +70 ‰ (Zhang et al., 2018). $\delta^{18}O_{NO3-}$ values in manure and sewage are below 15 ‰, while for NO_3^- originating from soil, $\delta^{18}O_{NO3-}$ varies between −5 ‰ and +5 ‰ (Kendall, 1998) (Table 2, Fig. 4).

The NO_3^- isotope signature found in groundwater or surface water is usually a mixture of various sources in changing proportions, e.g. N from fertilisers and mineralised N from soil organic matter (Gautam and Iqbal, 2010). Moreover, in view of the complex reaction processes including NO_3^- transformations (such as nitrification, denitrification and mineralisation – see Section 5) associated with isotopic fractionation that lead to altered $\delta^{15}N$ and $\delta^{18}O$ values, it is often difficult to identify the exact source of NO_3^-. However, with sophisticated calculation tools, both the mixing and fractionation processes can be combined to determine the possible ranges of different NO_3^- sources based on N and O isotope analyses (Lewicki et al., 2022).

4.3. Nitrite

Nitrite (NO_2^-), a crucial intermediate in the N cycle, has been assumed to be thermodynamically unstable and generally does not accumulate in the environment, hence it is often difficult or even impossible to detect and analyse. However, a higher NO_2^- concentration of 0.009 mg N L^{-1} has been detected in urban rivers, exceeding the limits of the European Water Framework Directive (EU WFD) (Sebilo et al., 2019). Significant amounts of NO_2^- may also be detected in soils, especially in phases of intensive denitrification (Lewicka-Szczebak et al., 2021). Adding the isotopic information of NO_2^- may provide crucial clues for deciphering N-transformation processes since, as a very reactive and short-lived compound, it provides insight into reactions as they happen (Casciotti, 2016).

NO_2^- in water and soil is a very unstable reactive compound. Therefore, it undergoes very dynamic exchange and is not usually directly added to the environment from particular sources, but rather is produced as an intermediate compound in numerous microbial pathways. Processes of NO_2^- formation include NO_3^- reduction during denitrification, NH_4^+ oxidation in autotrophic nitrification and organic N oxidation during heterotrophic nitrification. NO_2^- consumption occurs during NO_2^- reduction to NO or N_2O, and NO_2^- oxidation to NO_3^- (Buchwald and Casciotti, 2013; Lewicka-Szczebak et al., 2021). These sources and sinks are characterised by specific isotopic values and have been applied to identify the origins of NO_2^- and improve understanding of N transformation (see Section 5).

4.4. N gases

Besides mineral nitrogen forms, gaseous nitrogen forms are also an important part of N cycling. Numerous nitrogen transformations are associated with the gaseous products NO, N_2O and N_2.

N_2O emissions have increased significantly in recent years with enhanced agricultural practices (Van Groenigen et al., 2015; Zhang et al., 2020b). N_2O can be released as a by-product or intermediate product during four main microbiological processes: bacterial denitrification, fungal denitrification, nitrifier denitrification and nitrification. Co-denitrification and chemo-denitrification may also be additional sources of N_2O (Fig. 2). The $\delta^{15}N^{SP}$ values are characteristic of particular N_2O production pathways and are independent of substrate isotopic signatures (Toyoda et al., 2017; Yu et al., 2020b). Furthermore, $\delta^{18}O_{N2O}$ values also add valuable information about N_2O sources and sinks, and all three N_2O isotopic signatures can be combined together into a model to allow quantification of the contribution of different N_2O production pathways and the progress of its reduction (Lewicka-Szczebak et al., 2020; Lewicki et al., 2022).

NO is a very reactive intermediate product and only a very few studies have so far managed to analyse this compound. Nevertheless, these analyses can provide further insight into pathways of N gas emissions (Yu and Elliott, 2017, 2021) and can potentially be used for quantification of abiotic vs. microbial gas production pathways, differentiation between NH_4^+ oxidation and NO_3^- reduction processes, and complete tracking of the relationship between NO_2-NO-N_2O in particular pathways.

N_2 is the final product of denitrification, produced by N_2O reduction. Although it is an environmentally inert gas, it is an important component of the N budget, but its emission cannot be measured directly in natural conditions due to the high atmospheric background. N_2 flux can be quantified in N_2-free laboratory incubations or with indirect methods applying ^{15}N tracing or N_2O isotope studies (Lewicka-Szczebak et al., 2017).

Table 2
Overview of processes, sources and sinks influencing isotopic signatures $\delta^{15}N$ and $\delta^{18}O$ in N cycling. The isotopic signatures of NO_3^-,NO_2^-, NH_4^+ and N_2O, produced due to particular pathways, are shown in the 'source' column, and the isotopic fractionation associated with particular consumption processes is shown in the 'sink' column.

Process	Source				Sink						Citation
	$\delta^{15}N$ [‰]		$\delta^{18}O$ [‰]		$^{15}\varepsilon$ [‰]		$^{18}\varepsilon$ [‰]		$\delta^{15}N^{SP}$ [‰]		
	min	max	min	max	min	max	min	max	min	max	
NITRATE											
Rain	−10	+9	+20	+70							(Sharp, 2007; Zhang et al., 2018)
Synthetic fertilisers	−3	+3	+22	+24							(Kendall, 1998; Xue et al., 2009)
Manure, sewage	+3	+35	0	+15							(Kendall, 1998; Widory et al., 2005; Xue et al., 2009)
Soil N_{org}	+3	+8	−5	+5							(Kendall, 1998; Kendall and Aravena, 2000)
Autotrophic nitrification ($NH_4^+ \rightarrow NO_3^-$)	−37	−32	−9	+6							(Buchwald et al., 2012; Mariotti et al., 1981), this study calculations*
Heterotrophic nitrification ($N_{org} \rightarrow NO_3^-$)	+5	+12	+7	+25							(Spoelstra et al., 2007), this study calculations*
Nitrate reduction ($NO_3^- \rightarrow NO_2^-$)					−50	−2	−23	−5			(Granger et al., 2008; Kritee et al., 2012; Wang et al., 2018)
Bacterial denitrification ($NO_3^- \rightarrow N_2O$)					−53	+2					(Lewicka-Szczebak et al., 2014; Rohe et al., 2017)
Fungal denitrification ($NO_3^- \rightarrow N_2O$)					−46	−31					(Rohe et al., 2014, 2017)
Autotrophic denitrification ($NO_3^- \rightarrow NO_2^-$)					−13	−2	−11	−5			(Hosono et al., 2015; Liu et al., 2021)
DNRA ($NO_3^- \rightarrow NO_2^-$)					−40	−7					(Asamoto et al., 2021)
NITRITE											
Autotrophic nitrification ($NH_4^+ \rightarrow NO_2^-$)	−40	−14	−3	+14							(Buchwald et al., 2012; Casciotti et al., 2003; Liu et al., 2021), this study calculations*
Nitrite oxidation during nitrification ($NO_2^- \rightarrow NO_3^-$)					+7	+13	+5	+5			(Casciotti, 2009; Liu et al., 2021)
Heterotrophic nitrification ($N_{org} \rightarrow NO_2^-$)	−2	−1	+24 *	+25							(Möbius, 2013; Spoelstra et al., 2007), this study calculations*
Nitrate reduction ($NO_3^- \rightarrow NO_2^-$)	−25	−5	as nitrate sources								(Granger et al., 2008; Knöller et al., 2011; Lewicka-Szczebak et al., 2016; Rohe et al., 2014)
Nitrite reduction during denitrification ($NO_2 \rightarrow NO$)					−22	−8	−6	−2			(Martin and Casciotti, 2016)
Anammox ($NO_2^- \rightarrow N_2$)					−20	−10					(Magyar et al., 2021)
Nitrite oxidation during anammox ($NO_2 \rightarrow NO_3^-$)					+16	+43					(Brunner et al., 2013; Magyar et al., 2021)
AMMONIUM											
Rain	−13	+2									(Li et al., 2007)
Synthetic fertilisers, manure	−7	+5									(Kendall, 1998; Vitòria et al., 2004; Wassenaar, 1995)
Sewage	+5	+9									(Cole et al., 2006; Liu et al., 2006; Robertson et al., 2012)
Soil N_{org} mineralisation	−2	−1									(Möbius, 2013)
Nitrogen fixation	−4	−1									(Unkovich, 2013)
Autotrophic nitrification ($NH_4^+ \rightarrow NO_2^-$)					−40	−14					(Casciotti et al., 2003; Liu et al., 2021)
Autotrophic nitrification ($NH_4^+ \rightarrow N_2O$)					−64	−47					(Pérez et al., 2006; Yoshida, 1988)
Anammox ($NH_4^+ \rightarrow N_2$)					−32	−19					(Brunner et al., 2013; Magyar et al., 2021)
N_2O											
Nitrification ($NH_4^+ \rightarrow N_2O$)	−64	−47	+21	+26					+30	+39	(Frame and Casciotti, 2010; Pérez et al., 2006; Sutka et al., 2006; Yoshida, 1988; Yu et al., 2020b)
Bacterial denitrification ($NO_3^- \rightarrow N_2O$)	−53	+2	+7	+47					−8	+4	(Lewicka-Szczebak et al., 2014; Rohe et al., 2017; Sutka et al., 2006; Yu et al., 2020b)
Fungal denitrification ($NO_3^- \rightarrow N_2O$)	−46	−31	+42	+55					+28	+40	(Maeda et al., 2015; Rohe et al., 2014, 2017; Sutka et al., 2008; Yu et al., 2020b)
Nitrifier denitrification ($NH_4^+ \rightarrow N_2O$)	−61	−53	+8	+11					−14	−8	(Frame and Casciotti, 2010; Sutka et al., 2004; Yu et al., 2020b)
Hydroxylamine oxidation ($NH_2OH \rightarrow N_2O$)	−26	+6	+23	+24							(Heil et al., 2014; Sutka et al., 2003, 2004, 2006)
NO reduction during fungal denitrification ($NO \rightarrow N_2O$)	+14	+14									(Yang et al., 2014)
N_2O reduction due to denitrification ($N_2O \rightarrow N_2$)					−11	−2	−25	−5	−8	−3	(Yu et al., 2020b)

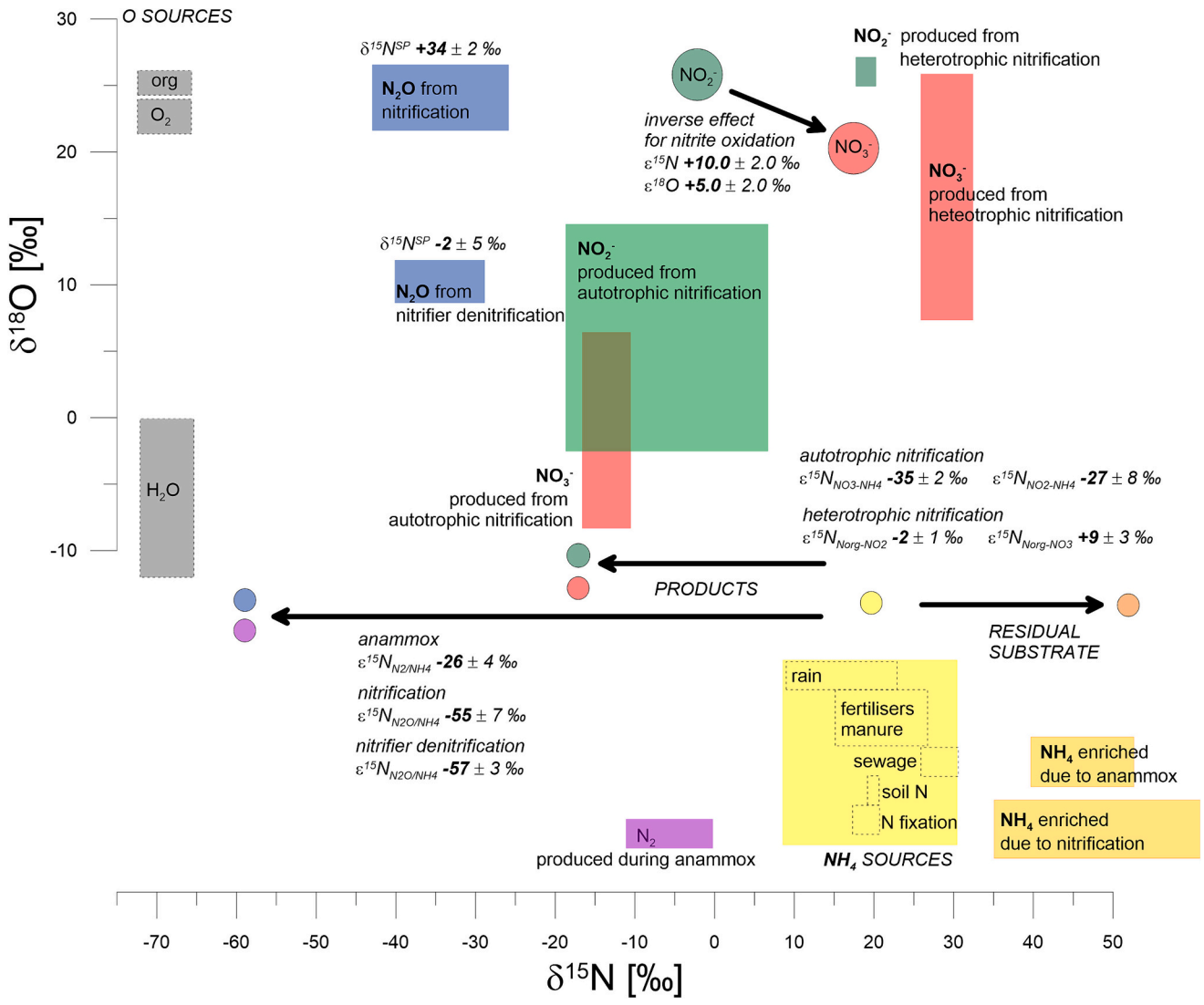

Fig. 3. Graphical summary of isotope effects during nitrification processes. The graph shows isotopic characteristics of NH_4^+ initial sources (yellow rectangles), residual NH_4^+ after its partial oxidation in nitrification or anammox processes (orange rectangles), and the products of nitrification and anammox processes: NO_2^- (green rectangles), NO_3^- (red rectangles), N_2O (blue rectangles) and N_2 (purple rectangle). The graphical illustration of the inverse N isotope effect of NO_2^- oxidation in the course of nitrification processes is presented by the circles. Respective values with citations are summarised in Table 2. Grey rectangles illustrate the oxygen sources for nitrification products (O_2, H_2O and organic matter (org)).

5. Microbial N transformations

N and O stable isotope signatures may be a useful tool for assessing different N sources. However, due to the isotopic fractionation associated with microbial processes (Fig. 2), the original isotopic signature, resulting from a mixing of various sources, can be significantly altered. Therefore the isotope effects of particular N transformations have to be taken into account. Microorganisms usually prefer light isotopes (^{14}N, ^{16}O), resulting in residual substrate enrichment in ^{15}N and ^{18}O. Consequently, reservoirs, including organic and inorganic N, are found enriched in ^{15}N as a result of microbial transformations (Gebus and Hałas, 2015), and the magnitude of this enrichment depends on the consumption progress.

For different microbial processes, the isotope effects are very variable, and thus a comprehensive knowledge of isotope effects for each N transformation process is critical for adequate interpretation of ^{15}N isotope patterns in the environment. The ability to differentiate between various microbial pathways responsible for N transformation is important for improved model developments and more adequate prediction and mitigation of possible N losses to the environment.

In this section, the general biochemistry of the microbes involved in the particular process is described for each pathway, and the associated isotope effects are summarised for N and O isotopes for each main N compound in the N cycle (NO_3^-, NO_2^-, NH_4^+ and N_2O). The overall summary of the isotope effects for all processes can be found in Table 2. Graphical presentations of the isotope effects associated with nitrification and denitrification processes are presented in Figs. 3 and 4, respectively.

5.1. Nitrification

5.1.1. Autotrophic nitrification

Nitrification (autotrophic nitrification) – a central process in the global N cycle – represents a two-step oxidation of NH_3 to NO_3^- via NO_2^- (Fig. 1). Nitrification is carried out by soil microorganisms generally at low pH, with oxic conditions, low to intermediate soil moisture and high C/N (Gebus and Hałas, 2015).

These two steps of nitrification are performed by distinct groups of microorganisms. Most nitrification processes occur aerobically, where ammonia is oxidised to NO_2^- via intermediate hydroxylamine (NH_2OH)

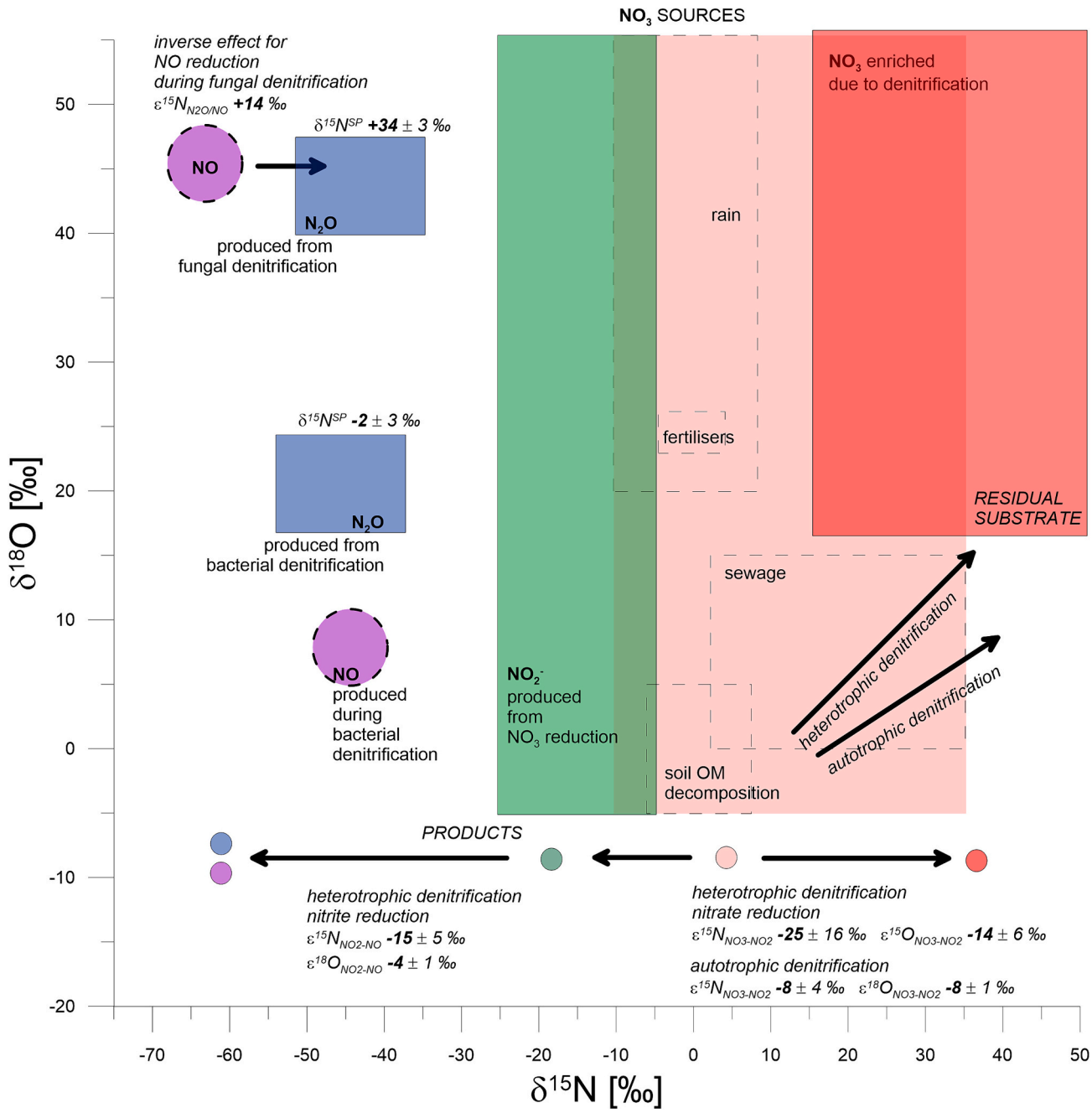

Fig. 4. Graphical summary of isotope effects during denitrification processes. The graph shows isotopic characteristics of initial sources of NO_3^- (light red rectangles), residual NO_3^- after its partial reduction during denitrification (dark red rectangles), and the products of denitrification processes: NO_2^- (green rectangles), N_2O (blue rectangles) and NO (purple circles; the values have been determined in very few studies to date, therefore dashed lines indicate the uncertainty of these values). Respective values with citations are summarised in Table 2.

by ammonia-oxidising bacteria (AOB) and archaea (AOA), catalysed by the enzyme ammonia monooxygenase (Bernhard, 2010). They are primarily chemolithoautotrophic ammonia oxidisers that belong to the groups *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosovibrio* and *Nitrosolobus* (Gebus and Hałas, 2015):

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (5)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (6)$$

Furthermore, oxidation of NO_2^- to NO_3^-, catalysed by the NH_2OH oxidoreductase enzyme, is performed by nitrite-oxidising bacteria which are relative autotrophs and belong to the genus *Nitrobacter* and *Nitrospira*:

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (7)$$

However, if NH_2OH is not fully converted to NO_2^- during nitrification, N_2O can be produced. This is facilitated by enzymes such as nitrite reductase (NIR) and nitric oxide reductase (NOR), which convert NO_2^- to NO and then to N_2O (Butterbach-Bahl et al., 2013).

Nitrification has shown a larger N isotope effect than other transformation processes in the N cycle, leading to the most ^{15}N-depleted species such as NO, N_2O, NO_2^- and NO_3^- (Mooshammer et al., 2020). NO_3^- produced due to nitrification is therefore characterised by a low $\delta^{15}N$.

One of the earliest studies on nitrification isotope effects (Mariotti et al., 1981) documented $^{15}\varepsilon_{NO3-/NH4+}$ of −34.7 ± 2.5 ‰ during

nitrification in pure cultures of *Nitrosomonas europaea*. Later studies of *Nitrosomonas europaea* (Casciotti et al., 2003) showed similar isotope effects of $^{15}\varepsilon_{NO2\text{-}/NH4+}$, ranging from −38 ‰ to −32 ‰, while *N. marina, N.* sp. *C-113a* and *Nitrosospira tenuis* showed comparatively smaller isotope effects of $^{15}\varepsilon_{NO2\text{-}/NH4+}$ in between −25 ‰ and −14 ‰ (Casciotti et al., 2003; Liu et al., 2021), which are similar to ammonia-oxidising archaea (AOA) from the North Pacific Ocean with $^{15}\varepsilon_{NO2\text{-}/NH4+}$ ranging from −41 ‰ to −13 ‰ (Santoro et al., 2011). Possible explanations causing variations in isotope effects may include differences in experimental conditions, such as pH and initial NH_4^+ concentrations. It was shown that at a high NH_4^+ concentration (1 mM) and pH values of between 6.5 and 8.5, $^{15}\varepsilon_{NO2\text{-}/NH4+}$ values were between −33.1 ‰ and −27.1 ‰ (substrate consumption) and between −35.5 ‰ and −31.2 ‰ (product formation) (Liu et al., 2021), which resembled the isotopic effects of *N. europaea* (−38.2 ‰) and *N. eutropha* (−32.8 ‰) (Casciotti et al., 2003) (Table 2).

The last nitrification step, NO_2^- oxidation to NO_3^-, is associated with an inverse isotope effect where the substrate NO_2^- is depleted in ^{15}N compared with the product (NO_3^-), and $^{15}\varepsilon_{NO3\text{-}/NO2\text{-}}$ ranges from +6.5 ‰ to +11.1 ‰ (Liu et al., 2021), which correlates with nitrite-oxidising bacteria *Nitrospira inopinata* (+9.5 ‰) (Buchwald and Casciotti, 2010; Jacob et al., 2017). Furthermore, for *Nitrococcus mobilis,* a marine nitrite-oxidising bacterium, a unique inverse kinetic isotope effect $^{15}\varepsilon_{NO3\text{-}/NO2\text{-}}$ of +12.8 ‰ was observed. Buchwald and Casciotti (2010) determined that the $^{18}\varepsilon_{NO3\text{-}/NO2\text{-}}$ effect for three nitrite-oxidising bacteria ranges from +1 ‰ to +8 ‰. Consequently, during NO_2^- oxidation, the $\delta^{15}N_{NO2\text{-}}$ and $\delta^{18}O_{NO2\text{-}}$ values of the residual NO_2^- become progressively depleted in ^{15}N and ^{18}O as the reaction proceeds. These results indicate inverse isotope fractionation at the enzyme level, and NO_2^- oxidation is therefore fundamentally different from all the other microbial processes in which N isotope fractionation has been studied. The unique kinetic isotope effect for NO_2^- oxidation should help improve understanding of its role in the cycling of NO_2^- in ocean suboxic zones, and other environments in which NO_2^- accumulates (Buchwald and Casciotti, 2013). This inverse effect can also be helpful in detecting or confirming the leading role played by the nitrification process in forming NO_2^- and NO_3^-. However, importantly, when the whole NO_2^- pool is oxidised to NO_3^-, there should be no difference in isotopic signatures between NO_2^- and NO_3^- produced in nitrification processes, as observed in the reported experimental ranges (Fig. 3). However, in the event that NO_2^- is not completely oxidised, the residual NO_2^- pool is depleted in heavy isotopes due to the inverse isotope effect during NO_2^- oxidation.

NO_3^- produced due to nitrification is characterised by low $\delta^{18}O$ values as a result of oxygen incorporation partly from the ambient water (2 atoms) and from atmospheric oxygen (1 atom). It is usually assumed that with the known values of $\delta^{18}O_{O2}$ in the atmosphere (+23.5 ‰) (Moore et al., 2006) and $\delta^{18}O_{H2O}$ in water, theoretical $\delta^{18}O_{NO3\text{-}}$ values ranging mostly between −10 ‰ and +10 ‰ can be calculated (assuming $\delta^{18}O_{H2O}$ of between −26 ‰ and +3 ‰) (Nikolenko et al., 2018). However, the process of water and oxygen incorporation is also associated with isotope effects linked to ammonia oxidation ($^{18}\varepsilon_{NO2\text{-}/NH4+}$) and further to NO_2^- oxidation ($^{18}\varepsilon_{NO3\text{-}/NO2\text{-}}$). Moreover, oxygen isotope exchange with water (x) during ammonia oxidation may also impact the final $\delta^{18}O_{NO2\text{-}}$ and $\delta^{18}O_{NO3\text{-}}$ values, according to the following equation (Buchwald et al., 2012):

$$\delta^{18}O_{NO2-} = \frac{1}{2}*\left(\delta^{18}O_{O2} + \delta^{18}O_{H2O} + {}^{18}\varepsilon_{NO2/O}\right)*(1-x) + \left(\delta^{18}O_{H2O} + {}^{18}\varepsilon_{NO2/H2O}\right)*x \quad (8)$$

where x is the O-isotope exchange with water during ammonia oxidation, which can be very variable depending mostly on the time of NO_2^- accumulation (Buchwald et al., 2012). $^{18}\varepsilon_{NO2/O}$ represents the summary isotope effect associated with oxygen and water incorporation, and varies from −22.7 ‰ to −11.4 ‰ with a mean of about −20 ‰ (Buchwald et al., 2012). $^{18}\varepsilon_{NO2/H2O}$ represents the isotope equilibrium effect during isotope exchange with water, which is 13.6 ‰ for 20 °C (Buchwald and Casciotti, 2013). During further oxidation of NO_2^- to NO_3^-, there is a second step of H_2O incorporation, with very variable $^{18}\varepsilon_{NO3\text{-}NO2}$ ranging from −27.2 ‰ to −0.8 ‰, and a mean of approximately −9 ‰ (Buchwald and Casciotti, 2013). During this last oxidation step, the exchange of oxygen atoms was found to be negligible (Buchwald and Casciotti, 2010), hence:

$$\delta^{18}O_{NO3-} = \frac{2}{3}\,\delta^{18}O_{NO2-} + \frac{1}{3}\left(\delta^{18}O_{H2O} + {}^{18}\varepsilon_{NO3-H2O}\right) \quad (9)$$

In summary, NO_3^- formed due to nitrification incorporates O isotopes from water, up to 100 % in the event of full O exchange during ammonia oxidation, with admixture of up to 33 % atmospheric oxygen. The isotope effects during O incorporation, however, result in isotope depletion in ^{18}O in the incorporated oxygen, whereas equilibrium O exchange with water results in enrichment in ^{18}O in relation to ambient water. Finally, with known values of $\delta^{18}O_{O2}$ in atmosphere (+23.5 ‰) and $\delta^{18}O_{H2O}$ in water (assuming values between mean ocean $\delta^{18}O_{H2O}$ of 0 ‰ and mean terrestrial $\delta^{18}O_{H2O}$ of −10 ‰), and taking mean isotope effects into account, $\delta^{18}O_{NO2\text{-}}$ and $\delta^{18}O_{NO3\text{-}}$ would range from −3 ‰ and −9 ‰ (when no O exchange occurs) and up to +14 ‰ and +6 ‰ (for complete O exchange) respectively, which is in line with the values also roughly estimated by Nikolenko et al. (2018) for nitrification-originated NO_3.

During bacterial nitrification, N_2O is formed as a by-product during the enzymatic oxidation of NH_2OH to NO_2^- (Fig. 2). Yoshida (1988) reported large fractionation of ^{15}N in *N. europaea* during NH_4^+ oxidation to N_2O, with $^{15}\varepsilon_{N2O/NH4}$ from −64 ‰ to −46.9 ‰. The largest isotope effects between NH_4^+ and N_2O were observed in soil incubation experiments and ranged from −111 ‰ to −102 ‰ (Pérez et al., 2006), but this study defined nitrification as all processes not inhibited by acetylene addition (see Section 3.2 for the acetylene inhibition method), which can result in different values. Hence, these values were not included in the summary table or graphs, in line with a previous review by Denk et al. (2017).

Characteristic $\delta^{15}N^{SP}$ values for N_2O originating from nitrification processes range from 31.4 ‰ to 38.7 ‰ (Frame and Casciotti, 2010; Sutka et al., 2006). $\delta^{18}O_{N2O}$ values determined in nitrifier cultures incubated with NH_4^+ were close to those of atmospheric oxygen at 23.5 ± 1.3 ‰ (Frame and Casciotti, 2010; Sutka et al., 2006), with only a subtle isotope effect resulting in an $^{18}\varepsilon_{N2O/O2}$ value of −2.9 ‰. Hence, for this process, $\delta^{18}O_{N2O}$ values of 23.5 ± 3 ‰ can be assumed (Fig. 3).

Pure cultures studies with *N. europaea*, *N. multiformis* and *M. trichosporium* were applied to determine the isotope effect of NH_2OH oxidation to N_2O ($\varepsilon_{N2O/NH2OH}$), which ranged from −5.7 ‰ to −2.0 ‰ (Sutka et al., 2006). This shows the much lower isotope effect for N_2O produced during NH_2OH oxidation than during NH_4^+ oxidation, indicating a strong isotope effect for NH_4^+ oxidation to NH_2OH (Denk et al., 2017). $\delta^{15}N^{SP}$ values of N_2O originating from NH_2OH oxidation are in the same range as for NH_4 oxidation, ranging from 32.5 ‰ to 35.6 ‰, and do not show a significant difference between species (Sutka et al., 2006). $\delta^{18}O_{N2O}$ is higher when NH_2OH is used as a substrate, varying from 38.6 ‰ to 39.7 ‰ (Sutka et al., 2006), which is probably a result of one more ^{18}O-enriched O atom in the NH_2OH substrate.

AOA produce N_2O with different isotopic characteristics. $^{15}\varepsilon_{N2O/NH4}$ of −16 ‰ to −6.2 ‰ is markedly lower than bacterially-produced N_2O, while $\delta^{18}O_{N2O}$ of 26.6 ‰ to 34.0 ‰ shows slightly higher values compared with AOB while $\delta^{15}N^{SP}$ of 28.9 ‰ to 30.3 ‰ is slightly lower (Jung et al., 2014; Santoro et al., 2011). The observed differences between isotopic signatures of N_2O produced by AOA and AOB are due to distinct N_2O production pathways. In AOB, NH_2OH (produced by ammonia monooxygenase AMO) is oxidised to NO_2^- by NH_2OH oxidoreductase (HAO), whereas AOA, as shown by the studies with the strain *Nitrosopumilus maritimus,* lack genes encoding the AOB-*Hao* complex, which suggests an alternative N_2O production process. *N. maritimus* was reported to generate a small amount of oxygen under anaerobic

conditions, probably by NO disproportionation. The oxygen produced is partly consumed in the ammonia oxidation pathway and respiratory chain, while some of it is released from the cells. The metabolic pathway of oxygen production might involve NO and N_2O as by-products (Wright and Lehtovirta-Morley, 2023).

5.1.2. Heterotrophic nitrification

Heterotrophic nitrification is the process of oxidation of organic N to NO_3^-. It is a much less studied process than autotrophic nitrification, but numerous recent studies indicate the significant or even dominant role of this process in N transformation and N_2O emissions (Lewicka-Szczebak et al., 2021; Zhang et al., 2015, 2023). Based on the ^{15}N tracing technique, where the substrates NH_4^+ and NO_3^- are both labelled with ^{15}N, a distinction can be made between heterotrophic and autotrophic nitrification thanks to the identification of the additional unlabelled N source in NO_3^- production (Zhang et al., 2011). Using this approach, it has been documented that heterotrophic nitrification may even be the dominant pathway for NO_3^- production in forest soils (Zhang et al., 2011), but it is not clear which microorganisms are involved in this pathway. Only the combination of the ^{15}N tracing technique and microbial inhibition methods allowed the determination of fungal and bacterial contributions to autotrophic and heterotrophic nitrification in forest soil (Zhu et al., 2015). The soils were labelled with $^{15}NH_4NO_3$ and $NH_4^{15}NO_3$ and treated with no antibiotics (control), a fungal inhibitor (cycloheximide) and a bacterial inhibitor (streptomycin) (see Section 3.2 for details of the SIRIN method). The results indicated that autotrophic nitrification was quite negligible, while heterotrophic nitrification was an important process in NO_3^- production. Fungal pathways rather than bacterial processes dominated heterotrophic nitrification (Zhu et al., 2015).

The isotope fractionation factors should be similar to autotrophic nitrification, however due to the different initial substrate, the two processes can potentially be distinguished due to the incorporation of distinct O and N isotopic values from organic matter (Spoelstra et al., 2007). NO_2^- analyses can be applied effectively to differentiate between the two types of nitrification (Lewicka-Szczebak et al., 2021). For NO_2^- produced during heterotrophic nitrification in marine sediments, ^{15}N enrichment was much lower than with autotrophic nitrification, with a mean $^{15}\varepsilon_{NO2/Norg}$ of about −2 ‰ (Möbius, 2013). Consequently, NO_3^- formed from heterotrophic nitrification will also show higher $\delta^{15}N$ values due to a small $^{15}\varepsilon_{NO2/Norg}$ isotope effect and inverse isotope effect for NO_2^- oxidation $^{15}\varepsilon_{NO2/NO3}$, finally giving $^{15}\varepsilon_{NO3/Norg}$ of ca. +5 ‰ to +12 ‰ (Table 2, Fig. 3).

Oxygen in NO_2^- formed due to heterotrophic nitrification should originate from the organic matter only. The oxygen isotopic composition of soil organic matter is similar to that of the plant matter from which it is derived, and its value should be slightly higher than in atmospheric oxygen, at about +24 ‰ to +25 ‰ (Fig. 3). By further oxidation to NO_3^-, an additional oxygen atom may be added from water or organic sources, consequently producing NO_3^- with two to three oxygen atoms derived from the organic N compound and only up to one from soil water:

$$\delta^{18}O_{NO3-} = 2/3\ \delta^{18}O_{org} + 1/3\ \delta^{18}\ O_{H2O} \quad (10)$$

As a result, NO_3^- originating from heterotrophic nitrification is expected to show significant enrichment in $\delta^{18}O$ compared with NO_3^- from autotrophic nitrification (Fig. 3), and the values can vary from +7 ‰ (assuming incorporation of one H_2O atom of minimal $\delta^{18}O$ value of -26 ‰) up to +25 ‰ when no water is incorporated (Table 2). It has been established that the O-atom exchange in the course of NO_2^- oxidation during nitrification processes is very low and does not exceed 3 % (Buchwald and Casciotti, 2010). Hence, the $\delta^{18}O$ signatures of NO_2^- and NO_3^- in the nitrification processes should follow Eqs. (9) and (10) for autotrophic and heterotrophic nitrification, respectively.

5.2. Denitrification

5.2.1. Bacterial denitrification

Denitrification plays a significant role in the natural attenuation of NO_3^- in groundwater systems (Nikolenko et al., 2018) and soils (Van Groenigen et al., 2015). Denitrification is the microbial dissimilatory stepwise reduction of NO_3^- to gaseous end products: N_2 via the intermediate NO, and N_2O:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (11)$$

Denitrification is an anaerobic process carried out by many facultative and strict anaerobic chemoorganotrophic bacteria in a stepwise enzymatic process. However, as some denitrifiers lack the N_2O reductase gene (*nosZ)* and N_2O reductase enzymes, the last denitrification step of N_2O reduction to N_2, denitrification is often incomplete, and N_2O is released as the final denitrification product (Harter et al., 2016). Certain other factors, including alkaline pH and high NO_3^- concentration, can affect microbial denitrification. In many denitrifying bacteria with low O_2 and NO_3^- availability, denitrification is initiated. Intermediates such as NO_2^- and NO are toxic to bacterial cells and should not accumulate under favourable conditions. At low concentrations (10-20 mM), NO_2^- can slow down bacterial activity at genomic level, while high NO_3^- concentrations (above 300 mM) can be detrimental to microbial denitrification and cause NO_2^- accumulation. For most denitrifying bacteria, the optimal pH is between 7.5 and 9.5. Low pH impacts reductase enzymes and slows down bacterial activity due to the formation of cytotoxic compound nitrous acid (HNO_2) from NO_2^-. At high pH, denitrification is observed to be slower with NO_2^- accumulation (van Groenigen et al., 2015).

Thanks to the bacterial denitrification method for NO_3^- analyses (Casciotti et al., 2002; Sigman et al., 2001), the dual isotope approach can be applied to analyse both $\delta^{15}N_{NO3-}$ and $\delta^{18}O_{NO3-}$ and thereby identify the denitrification processes and their role in N cycles (Osaka et al., 2018). During denitrification, ^{14}N and ^{16}O are preferentially converted to N_2O and N_2 by microorganisms, resulting in a significant enrichment of ^{15}N and ^{18}O in the remaining NO_3^-. This is usually expressed by the Rayleigh equations:

$$\Delta\delta^{15}N_{NO_3} \approx 1^{15}\varepsilon ln\left(C/C_0\right) \quad (12)$$

$$\Delta\delta^{18}O_{NO_3} \approx {}^{18}\varepsilon ln\left(C/C_0\right) \quad (13)$$

where C is the NO_3^- concentration and C_0 is the initial NO_3^- concentration. $\Delta\delta^{15}N_{NO3-}$ and $\Delta\delta^{18}O_{NO3-}$ are changes in the residual $\delta^{15}N_{NO3-}$ and $\delta^{18}O_{NO3-}$, respectively, at the reference point where $C = C_0$. $^{15}\varepsilon$ and $^{18}\varepsilon$ are the enrichment factors for ^{15}N and ^{18}O during denitrification, expressed as $\varepsilon_{S/S0}$ (see Section 2). These isotope effects for NO_3^- reduction are denoted as $^{15}\varepsilon_{NAR}$ and $^{18}\varepsilon_{NAR}$, respectively.

Isotope fractionation is influenced by the various circumstances and is sensitive to the denitrification rate, i.e. a larger isotope effect (more negative $^{15}\varepsilon$) is related to a lower denitrification rate (Mariotti et al., 1988). The NO_3^- reductase enzymatic experiments showed a mean $^{15}\varepsilon_{NAR}$ of −26.6 ± 0.2 ‰ (Karsh et al., 2012), whereas in pure culture bacterial studies much larger variations were observed, i.e. ranging from −30.5 ‰ to −5.4 ‰ (Barford et al., 1999; Granger et al., 2008; Knöller et al., 2011). The range from −15 ‰ to −10 ‰ is most representative for typical rates of cellular bacterial NO_3^- reduction (Kritee et al., 2012). In the sediment denitrification experiments, $^{15}\varepsilon_{NAR}$ ranged from −24.4 ‰ to −15.8 ‰ (Dahnke and Thamdrup, 2013; Sebilo et al., 2019), and a much wider range from −50 ‰ to −2 ‰ has been reported for soils (Lewicka-Szczebak et al., 2014, 2015; Mariotti et al., 1981; Menyailo and Hungate, 2006; Snider et al., 2009; Wang et al., 2018). Weaker fractionation with $^{15}\varepsilon_{NAR}$ ranging from −30 ‰ to −5 ‰ is typical for groundwater (Granger et al., 2008). A positive constant $^{18}\varepsilon/^{15}\varepsilon$ ratio

between 1/1.3 and 1/2.1 is associated with a decrease in NO_3^- content, and indicates the presence of denitrification (Osaka et al., 2018). Typically, denitrification has a greater enrichment effect on $\delta^{15}N$, and $\delta^{18}O$ is significantly less affected. This is most probably an indication of other reactions accompanying the denitrification process (Zhang et al., 2018) for NO_3^-, e.g. O-isotope exchange during NO_2^- re-oxidation (Kool et al., 2011). $^{18}\varepsilon/^{15}\varepsilon$ values for denitrification at watershed scales ranged from 0.50 to 0.76 (Osaka et al., 2018), while for in vitro studies $^{18}\varepsilon/^{15}\varepsilon$ for denitrifiers this figure was around 1 (Granger et al., 2008). In aquifers and marine sediment, $^{18}\varepsilon/^{15}\varepsilon$ values of isotopic fractionations during denitrification were measured to 0.88-1.04 and 0.78 respectively (Granger et al., 2008). The difference in $^{18}\varepsilon/^{15}\varepsilon$ values between field conditions and laboratory studies may be due to differences in the denitrification rate, which could lead to an erroneous estimation of $^{18}\varepsilon/^{15}\varepsilon$ under different conditions. Most probably, however, these variations are due to overprinting of the typical denitrification signal by other processes, such as nitrification or anammox (Granger and Wankel, 2016). Therefore, in natural environments, the individual N transformation should be always considered in conjunction with the whole complex N cycling. However, the processes can only properly be recognised when multiple compounds are analysed. This inconsistency in NO_3^- isotope signatures was explained by the mixing of multiple pathways by an isotope model proposed by Granger and Wankel (2016) distinguishing between denitrification, nitrification and anammox fluxes based on $\delta^{18}O$ and $\delta^{15}N$ analyses of NO_3^-, NO_2^- and NH_4^+.

NO_2^- that is formed in the process of denitrification, i.e. from NO_3^- reduction, shows depletion in ^{15}N compared with the original NO_3^-, according to the $^{15}\varepsilon_{NAR}$ values summarised above. The $\delta^{18}O$ values of the formed NO_2^- are similar to those of the original NO_3^- sources (Fig. 4) as a result of low oxygen fractionation between NO_3^- and NO_2^-. This is due to the cancellation of the intramolecular effect of about 30 ‰ associated with O branching (preferential disintegration of ^{16}O during NO_3^- reduction) (Casciotti et al., 2007) by the intermolecular effect when the NO_3^- pool is not completely consumed and ^{18}O-depleted NO_3^- molecules are preferentially consumed (Lewicka-Szczebak et al., 2016). This NO_2^- pool is often completely reduced further to NO, however through in case of intensive denitrification processes its accumulation is possible, and its isotopic signature is a joint result of its formation signature being changed by further reduction or reoxidation (Lewicka-Szczebak et al., 2021).

For NO_2^- reduction, there have been only a few studies to determine the N isotope effect for denitrification. Martin and Casciotti (2016) studied six strains of heterotrophic denitrifying bacteria to estimate N and O isotope fractionation effects for NO_2^- reduction. There are two discrete sets of nitrite reductase (NIR) enzymes, namely copper-based (Cu-NIR) enzymes and iron-based (Fe-NIR) enzymes encoded by the genes *nirK* and *nirS*, respectively. These enzymes, while reducing NO_2^- to NO gas, employ distinct metal centres – copper for Cu-NIR and iron for Fe-NIR – within their catalytic sites. These different metal centres may result in differences in N and O isotope effects. For denitrifier strains carrying the Cu-NIR, $^{15}\varepsilon_{NIR}$ was -22 ± 2 ‰ and $^{18}\varepsilon_{NIR} = -2 \pm 2$ ‰, while strains with Fe-NIR showed $^{15}\varepsilon_{NIR}$ of -8 ± 2 ‰ and $^{18}\varepsilon_{NIR}$ of -6 ± 2 ‰ (Martin and Casciotti, 2016). The strains showed a significant difference in isotope effects for NO_2^- reduction, probably associated with the difference in how the two enzymes bind NO_2^-.

N_2O is an intermediate product in denitrification. $^{15}\varepsilon_{N2O/NO3-}$ values determined in a pure denitrifier culture have shown a very wide range from −30.5 ‰ to +2.3 ‰ (Barford et al., 1999; Rohe et al., 2017; Toyoda et al., 2005). However, controlled soil incubation experiments dominated by N_2O production by bacterial denitrification (confirmed by $\delta^{15}N^{SP}$ values and ^{15}N tracing) show much lower values than pure culture studies, from −52.8 ‰ to −39.2 ‰ (Lewicka-Szczebak et al., 2014). These differences in $^{15}\varepsilon_{N2O/NO3-}$ between soil incubations, containing the whole microbial community, and pure culture studies, investigating separately one bacterial strain, are probably due to different N_2O production rates (N_2O reduction to N_2 was taken into account in these studies). $\delta^{15}N^{SP}$ values determined in pure culture studies showed a range from −7.5 ‰ to +3.7 ‰ (Rohe et al., 2017; Sutka et al., 2006), and the values obtained in the controlled soil incubation experiments fit within the range given by pure culture studies at values of between −4.7 ‰ and +1.7 ‰ (Lewicka-Szczebak et al., 2014). This confirms that the $\delta^{15}N^{SP}$ values typical for N_2O production from denitrification are very conservative signatures and are not affected by N_2O production rates (Lewicka-Szczebak et al., 2015). $\delta^{18}O_{N2O}$ values determined in pure culture studies show a wide range of values from 7.3 ‰ to 46.5 ‰ (Rohe et al., 2017; Sutka et al., 2006; Toyoda et al., 2005), probably due to variable O exchange with ambient water, depending on the bacterial strain. However, all bacterial strains show potential for O-isotope exchange, with most of them in fact displaying high exchange rates (Kool et al., 2011). Moreover, for naturally occurring soil communities, O exchange appears primarily to determine $\delta^{18}O_{N2O}$ values, with a quite stable isotope effect between water and the formed N_2O of $^{18}\varepsilon_{N2O/H2O}$ between 16.7 ‰ and 23.3 ‰ (Lewicka-Szczebak et al., 2016).

5.2.2. Fungal denitrification

Fungal denitrification may play an important role in the N cycle due to its potentially substantial contribution of N_2O gas emission in soil. However, an estimation of its actual role in natural systems is still lacking. Studies have indicated that in addition to bacteria, fungi are also capable of denitrification and N_2O production (Bollag and Tung, 1972; Mothapo et al., 2013; Shoun et al., 1992). Probably only a small number of fungi are capable of producing N_2O (Keuschnig et al., 2020), and to date there is practically no relevant information about the proportion of N_2O produced during fungal denitrification compared with that of bacteria (Rohe et al., 2021). It is assumed that fungal denitrification requires sub-oxic conditions, whereas bacterial denitrification is most effective in a fully anoxic atmosphere (Higgins et al., 2016). Some studies assume fungal denitrification to be a possibly dominant pathway of N_2O production, providing 34-42 % of total N_2O emissions (Aldossari and Ishii, 2021). Most fungi lack the N_2O reductase (NOS) enzyme that reduces N_2O to N_2 and hence, in contrast to bacterial denitrification, yield N_2O as the end product of fungal denitrification (Aldossari and Ishii, 2021; Shoun et al., 1992).

The characteristic isotope effects for N and O during NO_3^- reduction are similar to bacterial denitrification (Rohe et al., 2017). This process can mostly be distinguished based on the isotope signatures of the formed N_2O. Sutka et al. (2008) studied fungal denitrification in two cultures, *Fusarium oxysporum* and *Cylindrocarpon tonkinese*, and showed significant differences in $\delta^{15}N^{SP}$ values between fungal (36.9 ‰ to 37.1 ‰) and bacterial denitrification (0 ‰). Further investigation of the soil fungal strains *F. oxysporum, Trichoderma hamatum, Cylindrocarpon lichenicola, Fusarium solani fsp. Pisi* and *Fusarium decemcellulare* with either NO_3^- or NO_2^- as the electron acceptor confirmed fungal N_2O to have substantially higher $\delta^{15}N^{SP}$ values compared with bacterial N_2O (Rohe et al., 2014). Summarising all the studies on fungal N_2O production gives a $\delta^{15}N^{SP}$ range from 27.2 ‰ to 39.9 ‰ (Maeda et al., 2015; Rohe et al., 2014, 2017; Sutka et al., 2008) (Table 2). One study also indicated a lower $\delta^{15}N^{SP}$ value for one individual fungal species, which can be disregarded due to its very low N_2O production: *C. funicola* showed a $\delta^{15}N^{SP}$ value of 21.9 ‰, but ca. 100 times lower N_2O production with NO_2^- than other species, and no N_2O production at all with NO_3^- (Rohe et al., 2014), most probably due to its inability to perform anaerobic respiration (Rohe et al., 2020). Similarly, from a study of 70 fungal strains by Maeda et al. (2015), only the values of strains with significant N_2O production (>10 mg N_2O-N/g biomass) were taken into account, since some fungi species characterised by low N_2O production also indicated lower $\delta^{15}N^{SP}$ values. The $\delta^{15}N^{SP}$ values of the N_2O produced can discriminate between bacterial and fungal denitrification pathways, however the $\delta^{15}N^{SP}$ of fungal denitrification may overlap with other coexisting N_2O production pathways, especially nitrification (Yu et al., 2020b). Therefore, further complex analysis of $\delta^{15}N_{N2O}$, $\delta^{18}O_{N2O}$ and $\delta^{15}N^{SP}$ combined with microbial validation methods should provide an

approach for constraining the fungal contribution to N_2O production more effectively (Aldossari and Ishii, 2021; Rohe et al., 2021).

The $^{15}\varepsilon_{N2O/NO3}$ values were comparable with bacterial denitrification, ranging from −46 ‰ to −31 ‰, and the magnitude of this effect correlated with the denitrification rate (Rohe et al., 2014). The $^{15}\varepsilon_{N2O/NO2}$ values were smaller and ranged from −29.3 ‰ to −8.8 ‰ (Rohe et al., 2014; Sutka et al., 2008). In addition, the $\delta^{18}O_{N2O}$ of fungal denitrification was quite distinct from bacterial production pathways for N_2O production. $^{18}\varepsilon_{N2O/H2O}$ ranged from 42.0 ‰ to 55.1 ‰ and depended on the extent of oxygen isotope exchange between water and denitrification intermediates (Rohe et al., 2014, 2017). Sutka et al. (2008) reported a relatively minimal variation in $\delta^{18}O$ compared with $\delta^{15}N$ in N_2O produced by two fungal species, *C. tonkinese* and *F. oxysporum,* with $\delta^{18}O$ values of 31.5 ± 0.5 ‰ and 37.3 ± 1.3 ‰, respectively. This suggests that $\delta^{18}O$ could be a stable tracer for this particular process and can be applied to differentiate fungal denitrification from other pathways of N_2O production. Importantly, a significant difference in $\delta^{18}O$ in N_2O production via fungal denitrification and bacterial nitrification of NH_4^+ was observed. The differences in $\delta^{18}O$ between these processes can be attributed to variations in the isotopic composition of the oxygen source. During N_2O production through bacterial ammonia oxidation, oxygen originates from atmospheric oxygen and water. However, during denitrification, the oxygen atom is from NO_3^- or NO_2^-. This variation in the oxygen source contributes to differences in $\delta^{18}O$ between fungal denitrification and nitrification (Yu et al., 2020b).

Another intermediate of denitrification is NO, however few studies have examined NO isotopic signatures. Yang et al. (2014) studied the isotope effect in fungal denitrification with the enzyme P450-NOR during NO reduction to N_2O, and reported an inverse isotope effect for NO reduction with $^{15}\varepsilon_{N2O/NO}$ of 14.0 ± 1.6 ‰.

5.3. Nitrifier denitrification

Nitrifier denitrification is the process of NO_2^- reduction by AOB, with the oxidation of NH_3 to NO_2 followed by the reduction of NO_2^- to NO, N_2O and N_2. This is one of the major biochemical pathways for N_2O production, and possibly a major source of N_2O emission in soil (Kool et al., 2010; Wrage-Mönnig et al., 2018). Several AOB including *Nitrosomonas europaea*, *Nitrosospira* spp. have been studied extensively and found to produce N_2O, and may provide up to 80 % of total soil N_2O emissions (Shaw et al., 2006). *Nitrosospira briensis* was reported to produce N_2O partly through nitrifier denitrification (Wrage et al., 2004), while seven strains of *Nitrosospira* spp. produced N_2O completely through nitrifier denitrification (Shaw et al., 2006). Importantly, the nitrifier denitrification carried out by ammonia oxidisers clearly differs from the coupled nitrification and denitrification processes, where the denitrifying strains use the NO_3 produced by nitrifying strains. Nitrifier denitrification is most active in the environments with varying oxic and anoxic conditions, so far mostly documented for water treatment and soil fertilisation studies (Wrage-Mönnig et al., 2018). It may be also enhanced in C-limited environments and where there is large NO_2^- accumulation (Wrage-Mönnig et al., 2018). Important progress in distinguishing and quantifying nitrifier denitrification was achieved with dual-isotope labelling, applying the combined use of ^{18}O and ^{15}N isotope tracing (Wrage et al., 2005). This method applied in soil incubation studies indicated that nitrifier denitrification made a major contribution to NH_4^+-derived N_2O, while N_2O production as a by-product of nitrification was negligible (Kool et al., 2010).

N_2O produced through nitrifier-denitrification by *Nitrosomonas marina* indicated $\delta^{15}N_{N2O}$ of −56.9 ‰ ± 3.8 ‰, $\delta^{18}O_{N2O}$ of 8.4 ± 1.4 ‰ and $\delta^{15}N^{SP}_{N2O}$ of -10.7 ± 2.9 ‰ (Frame and Casciotti, 2010). Furthermore, *Nitrosomonas europaea* cell cultures with NO_2^- as the substrate (Sutka et al., 2004) produced $\delta^{15}N_{N2O}$ and $\delta^{18}O_{N2O}$ values of −34.0 ± 2.7 ‰ and 8.8 ± 1.4 ‰ respectively, and low $\delta^{15}N^{SP}_{N2O}$ of −0.8 ± 5.8 ‰. Similar values were observed for *Nitrosospira multiformis* cell suspensions with $\delta^{15}N_{N2O}$ and $\delta^{18}O_{N2O}$ of −22.9 ± 0.6 ‰ and 10.8 ± 0.5 ‰, respectively, while $\delta^{15}N^{SP}_{N2O}$ reached 0.1 ± 1.7 ‰ (Sutka et al., 2008), which was significantly different from that produced from NH_2OH, indicating a difference between nitrification and nitrifier denitrification pathways (Table 2). $^{15}\varepsilon_{N2O/NH4+}$ for nitrifier denitrification has been provided by only one pure culture study, and ranged from −60.7 ‰ to −53.1 ‰ (Frame and Casciotti, 2010) (Table 2).

5.4. Anammox

Anammox (anaerobic ammonium oxidation) plays a significant role in the N cycle. It is an autotrophic biological process mediating the conversion of NH_4^+ to N_2, while also generating NO_3^-. It occurs under anaerobic conditions and is performed by microorganisms belonging to the groups *Brocadia* (*B. anammoxidans and B. fulgida*), *Kuenenia* (*K. stuttgartiensis*) and *Scalindua* (*S. wagneri, S. brodae, S. sorokinii*) (Brunner et al., 2013; Gebus and Hałas, 2015; Magyar et al., 2021). The reaction for anammox can be expressed as:

$$1\ NH_4^+ + 1.3\ NO_2^- \rightarrow 1\ N_2 + 0.3\ NO_3^- \quad (14)$$

Microorganisms responsible for this process do not require any external organic compounds, unlike the denitrification bacteria (Gebus and Hałas, 2015), hence anammox is typical for low C conditions. Each of the molecules (NH_4^+, NO_2^-, and NO_3^-, N_2) links anammox to different processes in the N cycle. Stable isotope measurements are crucial for identifying sources and sinks of different molecules in the environment. Variations in $^{15}N/^{14}N$ in NH_4^+, NO_2^- and NO_3^- can provide information about different pathways, quantifying relative fluxes associated with the processes, fixed N and its removal from the ecosystem (Magyar et al., 2021).

Few studies have addressed the N isotope effects associated with the anammox metabolism and the potential impacts of N loss. Brunner et al. (2013) analysed *Kuenenia stuttgartiensis* cultures of anammox single cells and associated N isotope ratio effects in NH_4^+, NO_2^- and NO_3^- during the anammox reaction. Research results indicated preferential removal of ^{14}N from the NH_4^+ pool with an isotope effect $^{15}\varepsilon_{N2/NH4}$ of between −29.1 ‰ and −23.5 ‰, depending on factors controlling the anammox reaction reversibility. The study estimated a strong inverse kinetic N isotope effect for oxidation of NO_2^- to NO_3^- ($^{15}\varepsilon_{NO3/NO2}$ = +31.1 ± 3.9 ‰), with ^{15}N preferential transfer to a more oxidised compound, and normal N isotope fractionation for the reduction of NO_2^- to N_2 ($^{15}\varepsilon_{N2/NO2}$ = −16.0 ± 4.5 ‰), with ^{15}N preferential transfer to a more reduced compound. When anammox was exposed to environmental stress, an equilibrium N isotope effect between NO_3^- and NO_2^- ($^{15}\varepsilon_{NO3/NO2}$ = +60.5 ± 1.0 ‰) was also observed, which leads to enrichment in ^{15}N of the more stable compound, NO_3^- (Brunner et al., 2013).

Magyar et al. (2021) measured stable N-isotope composition of NH_4^+, NO_2^- and NO_3^- in wastewater cultivations of anammox bacteria, including members of Genera *Candidatus Brocadia*, *Ca. Kuenenia*, and *Ca. Jettenia*. For NO_2^- reduction to N_2, the N isotope enrichment factor $^{15}\varepsilon_{N2/NO2}$ was calculated to be −13.5 ± 3.7 ‰, indicating the presence of the anammox bacterial community. An inverse isotope effect was observed for oxidation of NO_2^- to NO_3^- with $^{15}\varepsilon_{NO3/NO2}$ ranging from +43 ‰ to +16 ‰, while for the reduction of NH_4^+ to N_2, $^{15}\varepsilon_{N2/NH4}$ was measured to be between −32 ‰ and −19 ‰. Kotajima et al. (2020) confirmed the inverse $^{15}\varepsilon_{NO3/NO2}$ isotope effect of $^{15}\varepsilon_{NO3/NO2}$ to be +20.6 ‰. These variations in isotope effects may be associated with differences in metabolism between different anammox species and increase the complexity of N-isotope interpretation. The contribution of NO_2^- oxidation associated with anammox processes impacts the $^{18}\varepsilon/^{15}\varepsilon$ ratio typical of denitrification processes, hence the shift of this ratio below 1 may be indicative of anammox processes.

5.5. DNRA

Although denitrification has been considered the primary process for removal of NO_3^- in the environment, a bacteria-mediated heterotrophic

process under anaerobic conditions may lead to dissimilatory NO_3^- reduction to NH_4^+ (DNRA) (Espenberg et al., 2018). Denitrification and DNRA can occur simultaneously and both can contribute to N_2O production. In recent years, a ^{15}N isotope tracing technique has been applied to quantify denitrification and DNRA pathways in nitrogen cycling studies, and even allows quantification of N_2O production, distinguishing between anammox and coupled DNRA-anammox and including the production of N_2 by anammox (Salk et al., 2017; Song et al., 2016; Valiente et al., 2021).

Dissimilatory NO_3^- reduction to NO_2^-, which represents the initial step in DNRA and denitrification pathways, is catalysed by two different enzymes, periplasmic enzyme NAP and cytosolic enzyme NAR, showing a similar N isotope fractionation ($^{15}\varepsilon_{NO2/NO3}$) from −39.8 ‰ to −11.4 ‰ for NAP and from −31.6 ‰ to −6.6 ‰ for NAR (Asamoto et al., 2021). In addition, *Rhodobacter sphaeroides* and *Sulfurimonas gotlandica* were examined for NAP and NAR reductases, and the ratio $^{18}\varepsilon/^{15}\varepsilon$ ranged from 0.57 to 0.68 and from 0.43 to 0.68, respectively (Frey et al., 2014; Granger et al., 2008; Treibergs and Granger, 2017).

Furthermore, Asamoto et al. (2021) examined the $^{18}\varepsilon/^{15}\varepsilon$ ratio in six different nitrate-reducing microorganisms catalysed by cytosolic and periplasmic nitrate reductases (NAR and NAP), or by both enzymes and mutants NarG and NapA, to analyse enzymatic differences for microbial pathways. The results indicated that the denitrifying bacterial strains *P. aeruginosa* and *P. denitrificans* (which cannot perform DNRA) showed a negligible NO_2^- accumulation, while strains *B. vireti*, *B. bataviensis*, *D. desulfuricans* and *S. loihica*, which are able to perform DNRA, accumulated NO_2^- during the study. For *P. aeruginosa* and *P. denitrificans* (having both NarG and NapA), $^{18}\varepsilon$ / $^{15}\varepsilon$ was estimated as 0.97 ± 0.02 and 0.92 ± 0.01 respectively. For *D. desulfuricans* and *S. loihica* (with only NapA) $^{18}\varepsilon/^{15}\varepsilon$ was estimated to be 0.63 ± 0.06 and 0.55 ± 0.01, respectively, while for *B. vireti* and *B. bataviensis* (with only NapG), $^{18}\varepsilon/^{15}\varepsilon$ was 0.64 ± 0.04 and 0.61 ± 0.06 respectively. The study indicated distinct isotopic signatures between the enzymes NAP and NAR reductases.

5.6. Nitrogen fixation

Biological nitrogen fixation is a vital process for assimilation of atmospheric molecular nitrogen (N_2) into NH_4^+. N_2-fixing organisms are termed as diazotrophs and are carried out by the enzyme nitrogenase. Diazotrophs can fix N_2 in a 'free-living' state, while some fix N_2 in association with plants or complex symbioses involving roots or stems (Unkovich, 2013).

The schematic equation can be summarised as:

$$N_2 + 16ATP + 8e^- + 8H + \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (15)$$

The quantification of biological nitrogen fixation, a crucially important biologically mediated process, relies significantly on stable isotope-based studies intricately linked with N transformations (Unkovich, 2013). For biological N_2 fixation by the nitrogenase enzyme in diazotrophs, differences in $\delta^{15}N$ values between atmospheric N_2 and fixed NH_4 can help improve understanding of the N_2-fixation process (Zhang et al., 2014). Buckley et al. (2007) analysed the *nifH* gene in diazotrophs responsible for N fixation in soil, and indicated three microbial groups capable of N fixation: one from the *Rhizobiales* group and two belonging to unclassified non-cultivated bacteria within the *Betaproteobacteria* and *Actinobacteria* groups.

Hoering and Ford (1960) conducted some of the earliest investigations into isotope effects during nitrogen fixation. Their study focused on the examination of isotope effects in *Azotobacter* cultures cultivated in a laboratory setting using nitrogen gas (N_2), but no significant isotope discrimination associated with nitrogen fixation was observed. However, later Macko et al. (1987) studied N_2 fixation-dependent *Anabaena* and reported $^{15}\varepsilon_{NH4/N2}$ values of between −2.4 ‰ and −2.2 ‰. Rowell et al. (1998) studied the isotope effects of *Azotobacter* or *Anabaena* dependent on molybdenum- or vanadium-associated nitrogenase, with reported values for vanadium nitrogenase $^{15}\varepsilon_{NH4/N2}$ values of −4.0 ‰ and −1.0 ‰ for molybdenum nitrogenase (Unkovich, 2013) (Table 2).

5.7. Abiotic processes

Other physical processes, such as sorption and desorption, can also modify the isotopic composition of N species without involving microorganisms, hence these are associated with minor isotope effects when compared with microbial processes (Kendall, 1998). The anion exchange favours retention of ^{14}N and ^{16}O in NO_3^- ions, while the cation exchange prefers the heavier isotope, ^{15}N, in the adsorbed fraction of NH_4^+ (Kendall and Aravena, 2000). Numerous chemical N transformations, including photochemical and thermochemical reactions, are characterised by similar substrates and products as the microbial transformations described above (Doane, 2017). Abiotic and biotic nitrogen transformations in the environment are interconnected. Both abiotic and biotic processes involve simultaneous transformations of carbon and nitrogen, particularly in the incorporation of inorganic nitrogen into organic matter and photochemical dissolution. Furthermore, organic nitrogen compounds resistant to microbial decomposition can readily be broken down by sunlight, suggesting that both pathways contribute to nitrogen mineralisation (Doane, 2017).

Chemodenitrification is most probably the abiotic process of the greatest significance in the environment. It is the abiotic reaction of Fe (II) and NO_2^-, leading to N_2O production, and is initiated by reactive nitrogen species during denitrification. Chemodenitrification may be influenced by the presence of Fe(II) produced by heterotrophic Fe(III)-reducing microorganisms and the availability of NO_2^-, produced during NO_3^- reduction by heterotrophic denitrifying bacteria (Otte et al., 2019).

$$4Fe^{2+} + 2NO_2^- + 5H_2O \rightarrow 4FeOOH + N_2O + 6H^+ \quad (16)$$

Nitrite-induced chemodenitrification in coastal marine sediment may contribute up to 15-20 % of N_2O production, while in sterilised sediment, abiotic chemodenitrification contributed to approximately 17.4 % of total N_2O (Otte et al., 2019). Furthermore, a shift in the microbial community composition in response to different amendments was observed. In Fe(II) and nitrate-amended conditions, active microorganisms *Defluviicoccus*, *Sulfurimonas* and *Arcobacter* were enriched, while Fe(II) and nitrite amendment led to the enrichment of active microorganisms including *Psychrilyobacter, Propionigenium, Bacillus, Thauera* and *Marinobacter*. This shows the clear response of diverse microbial groups and their involvement in Fe and N cycling (Otte et al., 2019).

Distinguishing between denitrification and chemodenitrification processes based on isotopic offsets with NO_2^- or NO_3^- substrates is challenging. N_2O isotopic signatures show variations, with $^{15}\varepsilon_{N2O/NO3}$ for denitrification of between 10 ‰ and 39 ‰ and for chemodenitrification $^{15}\varepsilon_{N2O/NO2}$ varying from 2 ‰ to 30 ‰, exhibiting overlapping ranges with similar effects for the O isotopic effect, $^{18}\varepsilon_{N2O/NO3}$ of −40 ‰ to −4 ‰ for denitrification and $^{15}\varepsilon_{N2O/NO2}$ of −30 ‰ to −17 ‰ for chemodenitrification, (Tischer et al., 2022; Visser et al., 2020).

Abiotic reactions involving nitrification intermediates such as hydroxylamine (NH_2OH) and nitrite (NO_2^-) are abundant in soils. Heil et al. (2014) conceptualised a model integrating both biotic and abiotic reaction mechanisms for the formation of N_2O, NO and gaseous nitrous acid (HONO). It is suggested that stable isotope techniques are valuable tools for distinguishing between biotic and abiotic processes. $\delta^{15}N^{SP}_{N2O}$ serves as a valuable tool for gaining insights into N_2O production processes. Results indicated the feasibility of purely abiotic reactions occurring across a broad pH range (3-8) through different mechanisms: the reaction of NH_2OH with NO_2^- at low pH, oxidation of NH_2OH by Fe^{3+} within pH 3-8, and Cu^{2+} catalysed autoxidation of NH_2OH at higher pH levels. The $\delta^{15}N$ and $\delta^{18}O$ of N_2O produced varied significantly under

different reaction conditions. $\delta^{15}N$ of N_2O partially mirrored the precursors $\delta^{15}N$- NH_2OH (−1.93 ‰) and NO_2^- (−27.0 ‰), while $\delta^{18}O$ of N_2O exhibited a similar pattern. In summary, all abiotic pathways exhibited a consistent $^{15}N^{SP}_{N2O}$ of approximately 35 ‰, and remained unchanged in variable pH and reaction conditions, indicating a stable characteristic of these pathways throughout the experiments. Soil experiments further highlighted the influence of factors such as pH, C/N ratio and manganese content on N_2O formation. While no evidence of N_2O decomposition via photolysis was found, the study shed light on the complex interplay between biotic and abiotic processes in N_2O emissions from soils.

Abiotic processes in nitrogen cycling are often overlooked compared with microbial-mediated transformations (Doane, 2017). Abiotic reactions such as nitrogen fixation, oxidation and reduction contribute significantly to nitrogen dynamics in various environments. For instance, the spontaneous photochemical fixation of dinitrogen may play a substantial role in nitrogen cycling, particularly in environments with limited biological fixation such as deserts. Abiotic and coupled biotic-abiotic processes are often disregarded, highlighting the need for a deeper understanding of nitrogen dynamics to control nitrogen efficiently in ecosystems and alleviate environmental consequences (Doane, 2017).

5.8. Combining isotope data and applying modelling approaches

Isotope data are certainly a powerful tool for identifying and tracking N transformations, however due to the complexity of isotope effects they are not straightforward to apply. The main challenge lies in taking into account all the processes that impact the isotopic signature of the particular compound, including various sources of different isotope characteristics and consumption processes. This can be done by applying simple isotope models concerning one compound, e.g. NO_3^- (Yu et al., 2020a), NO_2^- (Lewicka-Szczebak et al., 2021), NO (Yu and Elliott, 2021) or N_2O (Lewicka-Szczebak et al., 2020), or more complex ecosystem models adding isotope signatures to the calculation of biochemical fluxes for terrestrial and marine environments (Denk et al., 2019; Martin et al., 2019).

The simplest isotope one-compound models are based on so-called mapping approaches, where the dual isotopic signatures are jointly interpreted on a common plot, e.g. $\delta^{15}N$-$\delta^{18}O$ for NO_3^- (Zhang et al., 2020a), $\delta^{15}N$-$\delta^{18}O$ for NO_2^- (Lewicka-Szczebak et al., 2021) or $\delta^{15}N^{SP}$-$\delta^{18}O$ and $\delta^{15}N^{SP}$-$\delta^{15}N$ for N_2O (Yu et al., 2020b). Such dual isotope plots (as in Fig. 2 and Fig. 3) often allow identification of the processes occurring, but may also attempt their quantification by applying isotope mass balance equations for source partitioning (Zhang et al., 2020a) or isotope fractionation equations to determine the consumption progress (Lewicka-Szczebak et al., 2017). For such estimations it is important to provide the uncertainty estimation of the calculated source contributions or consumption progress including not only analytical or sampling uncertainty, but also the ranges of possible isotope effects for the particular processes. To achieve this, Bayesian statistics can be applied (Lewicki et al., 2022; Zhang et al., 2020a). New software applying a Markov-Chain Monte Carlo model for the stable isotope, called Fractionation And Mixing Evaluation (FRAME, malewick.github.io/frame), has recently been developed to enable simultaneous automatic source partitioning and consumption progress estimation based on isotopic data together with an uncertainty estimation (Lewicki et al., 2022). FRAME also allows for the integration of three isotopic signatures of one compound into the model, which is crucial for N_2O models by enabling complex interpretation based on $\delta^{18}O$, $\delta^{15}N$ and $\delta^{15}N^{SP}$ of N_2O (Lewicka-Szczebak et al., 2020; Lewicki et al., 2022). Most recently also TimeFRAME software was developed for interpretation of time series isotope measurements of trace gases (Harris et al., 2023).

Isotope data can be also included in biogeochemical models and used as an additional parameter constraining the calculated fluxes. A first coupled isotope and process-based modelling approach was proposed by Bai and Houlton (2009), incorporating soil N isotope signatures into the DAYCENT process-based model to determine gaseous nitrogen losses from soils. Furthermore, Denk et al. (2019) developed the Stable Isotope Model for Nutrient Cycles (SIMONE), which incorporated $\delta^{15}N$ of soil mineral nitrogen and N_2O into the Landscape DNDC process-based model. Similar approaches have also been proposed for marine N-cycling studies. Martin et al. (2019) developed a 3-D inverse N cycle model by coupling oceanographic measurements of NO_3^- and NO_2^- concentrations with their isotopic signatures, which helped quantify N cycling rates in oceanic oxygen-deficient zones more effectively.

6. Case studies combining isotope and microbial methods

The chapters above have described the microbiological background and isotope effects for the various N transformation pathways. Both microbiological analyses and isotopic studies have their limitations, and applied alone are often not able to distinguish correctly between, or identify, particular pathways. Microbial studies allow the identification of particular microbial strains, but even when applying molecular methods, determining their actual activity is challenging and exact information is not obtained about the compounds being produced or consumed, or about the magnitude of these processes. In contrast, isotopic studies allow an examination of the compounds' isotopic signatures and, with the known isotope effects, the processes occurring can be theoretically identified and even quantified. However, due to possible multiple N transformations occurring simultaneously and wide ranges in the possible isotope effects, there are often too many unknowns, and the isotope results will not give unambiguous answers. Therefore, a combination of isotopic and microbiological approaches can provide complementary information and is promising for distinguishing between various N transformation processes and estimating their rates by evaluating key microbial communities and determining possible N transformation pathways actually occurring in the ecosystem (Maeda et al., 2010; Németh et al., 2014; Snider et al., 2015). The microbially-mediated activity is further compared with stable isotope measurements, which can provide a cross-validation of the interpretation of the results of both approaches, and consequently provide more extensive insight into the complex N cycle and better assess the potential contribution of microbial communities in the removal of excess N from the environment. A short summary follows of some examples showing what can be achieved with a combined approach of this kind. Such complex studies still remain few in number (summarised in Table 3).

Early applications of a combined approach of this kind concerned N_2O source partitioning and the estimation of its reduction (Snider et al., 2015). N_2O isotopocule studies are an efficient tool for distinguishing between various N_2O source processes (bacterial and fungal denitrification, nitrifier denitrification and nitrification). With well-defined source isotopic values, the N_2O production pathways can even be quantified using isotope models applying the three isotope signatures of N_2O ($\delta^{15}N$, $\delta^{15}N^{SP}$ and $\delta^{18}O$) (Lewicka-Szczebak et al., 2020; Lewicki et al., 2022). However, some of the isotope characteristics overlap, such as $\delta^{15}N^{SP}$ values for nitrification and fungal denitrification, impeding the identification and quantification of some processes. For proper application of the isotope models, the processes that should be taken into account need to be selected. Based on the complementary approaches, it is possible to pre-determine which particular transformations should be taken into consideration, thereby constraining the assumptions for the isotope modelling approaches, resulting in more precise outputs.

Snider et al. (2015) proposed the application of molecular methods as a complementary approach to study N_2O production in agricultural soils, specifically in drought-stressed soils induced by heavy rainfall. Prior rainfall N_2O emissions were very low and originated from denitrification. Interestingly, the molecular detection of gene transcripts indicated the co-occurrence of nitrification and denitrification processes, but the isotope studies indicated denitrification as the N_2O-

Table 3
Functional gene analysis combined with isotope analysis applied to track N transformation processes in water and soil studies.

Target genes	Enzyme	Active process	Environment	Combined isotope analysis and molecular methods	Research findings	Citation
nirS	Nitrite reductase	Denitrification	Agricultural soil	$\delta^{15}N_{N2O}$, $\delta^{18}O_{N2O}$	Coexistence of nitrification and denitrification	(Snider et al., 2015)
nirK, *nirS* *nosZ* *amoA*	Nitrite reductase nitrous oxide reductase ammonia monooxygenase nitrite reductase	Denitrification, nitrification	Peat soil	$\delta^{15}N_{N2O}$, $\delta^{15}N^{SP}_{N2O}$, $\delta^{18}O_{N2O}$	Major role of incomplete denitrification in N_2O production, significant hybrid N_2O formation	(Masta et al., 2023)
nirK, *nirS* *nosZ* *amoA* *nrfA*	Nitrite reductase nitrous oxide reductase ammonia monooxygenase Nitrite reductase	Denitrification, nitrification, comammox, DNRA	Peat soil	$\delta^{15}N_{N2O}$, $\delta^{15}N^{SP}_{N2O}$, $\delta^{18}O_{N2O}$	Dominant bacterial denitrification, contribution of comammox and DNRA	(Masta et al., 2022)
nirS	Nitrite reductase	Denitrification	Terrestrial ecosystems	$\delta^{15}N_{NO3-}$, $\delta^{18}O_{NO3-}$,	Microbial denitrification consume plant available NO_3^-	(Lennon and Houlton, 2017)
nirK	Nitrite reductase	Denitrification	River	$\delta^{15}N_{N2O}$, $\delta^{18}O_{N2O}$	Denitrification responsible for N_2O production	(Thuan et al., 2018)
nosZ	Nitrous oxide reductase	Denitrification	Soil	$^{15}N_2O$ isotope pool dilution	Increased N_2O consumption by organic carbon addition	(Stuchiner and von Fischer, 2022)
Hzo	Hydrazine oxidoreductase	Anammox	Oxygen minimum zones (OMZ)	$\delta^{15}N_{NO3-}$, $\delta^{18}O_{NO3-}$,	Anammox major pathway for N_2 production in OMZ	(Pajares et al., 2019)
nrfA	Nitrite reductase	DNRA	Oxygen minimum zones (OMZ)	$\delta^{15}N_{NO3-}$, $\delta^{18}O_{NO3-}$,	Accumulation of NH_4^+, DNRA may enhance anammox process	(Pajares et al., 2019)

source process. Importantly, the indication of nitrifier activity with molecular methods does not necessarily mean significant N_2O production by nitrification processes since this is only a facultative by-product. Rainfall triggered N_2O emission in soil, with a rapid and very pronounced shift in $\delta^{18}O$ and $\delta^{15}N$ towards lower values, mostly characteristic of nitrifier-denitrification processes. Simultaneously, a significant change in gene abundances in microbial communities was detected. Increased abundance of nitrifier and denitrifier genes and a high level of archaeal ammonia monooxygenase (*crenamoA*) and bacterial nitrite reductase (*nirS*) transcripts appeared in the soil, indicating nitrifier-denitrification as a dominant N_2O emission process, and that both nitrification and denitrification were occurring simultaneously. This study is an example of the complementary application of molecular and isotopic tools, which helped adequately identify the dominant N_2O production pathway. Stable isotope analyses, although without $\delta^{15}N^{SP}_{N2O}$ data, appeared useful for tracing the pathways of produced N_2O, while molecular analyses provided the background knowledge on the N-cycling communities before, during and after the emission event. This information helped explain the changes in N_2O fluxes observed and reinforced the isotope-based conclusions. A similar study combining isotope and microbial approaches for soil N_2O emissions associated with changes in moisture has been performed for peat forest soil (Masta et al., 2023). Under flooded conditions, the isotopic signatures of N_2O and NO_3^- suggested the dominance of denitrification, which was supported by an increase in the gene transcripts *nirK*, *nirS* and *nosZ* after the flooding. The proportion of gene abundances of *nosZ,* responsible for N_2O reduction, and *nirK* and *nirS*, responsible for N_2O production, supported the occurrence of incomplete denitrification. For drier treatments, the N_2O isotopic signatures indicating a mixing of various processes and molecular analyses allowed the identification of bacterial and archaea nitrifiers as the major N_2O source besides denitrification (Masta et al., 2023). Another peatland study also revealed the importance of the nitrification comammox pathway, indicated by high archaeal and bacterial *amoA* gene copy numbers, and a significant contribution of DNRA with increased N_2O emission correlated with an increase in *nrfA* gene copies (Masta et al., 2022). A positive correlation between isotopic studies and molecular analyses was also reported by Lennon and Houlton (2017) in a study that combined stable isotope techniques with quantitative real-time qPCR to quantify denitrification processes across the terrestrial ecosystem. In short-term soil-incubation experiments, *nirS* gene abundance was significantly positive in soil samples, while NO_3^- depletion was associated with an increase in $\delta^{15}N_{NO3}$, *nirS* and *nirS*/16 rRNA, suggesting microbial denitrification and enzymatic activity across soil samples. In another very complex soil study, Zhang et al. (2021) applied combined molecular, inhibition and isotopic approaches to study the long-term effects of biochar amendment and explain the underlying mechanism responsible for the observed reduction in N_2O emission. From the decreased NO_2^- content and a decrease in the (*nirK* + *nirS* + *fungal nirK*):(*nosZ-I* + *nosZ-II*) ratio, a significant decrease in N_2O production by nitrifier denitrification and heterotrophic denitrification was indicated, which was in line with the isotopic results.

Surface waters are significant sources of N_2O emissions to the environment, and isotopic and microbial methods can be applied to both water-emitted and soil-emitted N_2O. Thuan et al. (2018) measured the isotopic signatures of N_2O, NH_4^+, NO_2^- and NO_3^- in the Tama River (Japan) along with functional gene abundances to identify N_2O emission and associated microbial pathways. From the results, $\delta^{15}N_{N2O}$ and $\delta^{18}O_{N2O}$ indicated that denitrification is dominant for N_2O production in the river. This positively correlated with significant gene abundances of *nirK* responsible for N_2O production, and lower gene abundances of *nosZ* and *amoA*. In lake water studies, Li et al. (2022) performed two in-depth studies on how different electron donors (glucose, sulfide and iron(II)) affect NO_3^- reduction and influence N_2O isotope fractionation (Li et al., 2022a), and how different carbon sources influence N_2O production in freshwater (Li et al., 2022b). The results of lake sediment incubations with different electron donors revealed that the iron-driven microbial denitrification has a different pattern in $\delta^{15}N^{\beta}$ values as a result of a greater contribution of NO reductase with quinols as the electron donor (*qNorB*) (Li et al., 2022a). The second study showed characteristic isotopic patterns in $\delta^{15}N^{SP}_{N2O}$ and $\delta^{18}O_{N2O}$ depending on the applied carbon source, caused by differences in NO reductase enzymes. Namely, the lowest $\delta^{18}O_{N2O}$ values were typical for *cNorB* and *qNorB* and the highest

$\delta^{15}N^{SP}_{N2O}$ values were for *cNorB* (Li et al., 2022b). These two studies helped explain different N_2O isotopic patterns with differences in the active enzymes of NO reductases.

Similar methods can also apply to groundwater studies. Amo et al. (2018) compared isotopic and microbiological information to characterise the denitrification process in a rock aquifer. Isotopic studies suggested the occurrence of a denitrification process in the groundwater, where $\delta^{15}N_{NO3}$ ranged between +10.4 ‰ and +26.9 ‰ and $\delta^{18}O_{NO3}$ was valued between +3.8 ‰ and +12.3 ‰. Microbiological analyses correlated with isotopic studies by detecting significant gene abundance of *nirS* and *nirK*, suggesting NO_2^- reduction. Furthermore, the *nrfA* gene responsible for DNRA and N_2O reductases *nosZI* and *nosZII* was reported (Amo et al., 2018).

Investigating oceanic oxygen minimum zones (OMZs), Pajares et al. (2019) explored the genetic potential of the *hzo* gene encoding anammox and the *nrfA* gene encoding DNRA. In deeper portions of OMZs, the *hzo* gene was abundant, while the *nrfA* gene increased where the NO_3^- concentration was highest. This indicates that DNRA may be an important process enhancing anammox in oxygen-deficient zones.

7. Outlook

Recent research on N cycling in the environment have added a considerable amount of information on the isotope effects associated with N transformations and on microbial and molecular analyses. With the constantly growing database of various processes and microorganisms involved in N cycling, it is increasingly challenging to combine different approaches and properly understand and interpret the results obtained. This review, with its summary of N transformations, associated isotope effects for all N compounds and possible molecular methods that can be applied, should help provide a better overview of the possible processes and ideas being developed in new research directions, including the links between isotopic and microbiological methods.

CRediT authorship contribution statement

Sushmita Deb: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Dominika Lewicka-Szczebak:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Lena Rohe:** Writing – review & editing, Writing – original draft.

Declaration of competing interest

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Data availability

Data will be made available on request.

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