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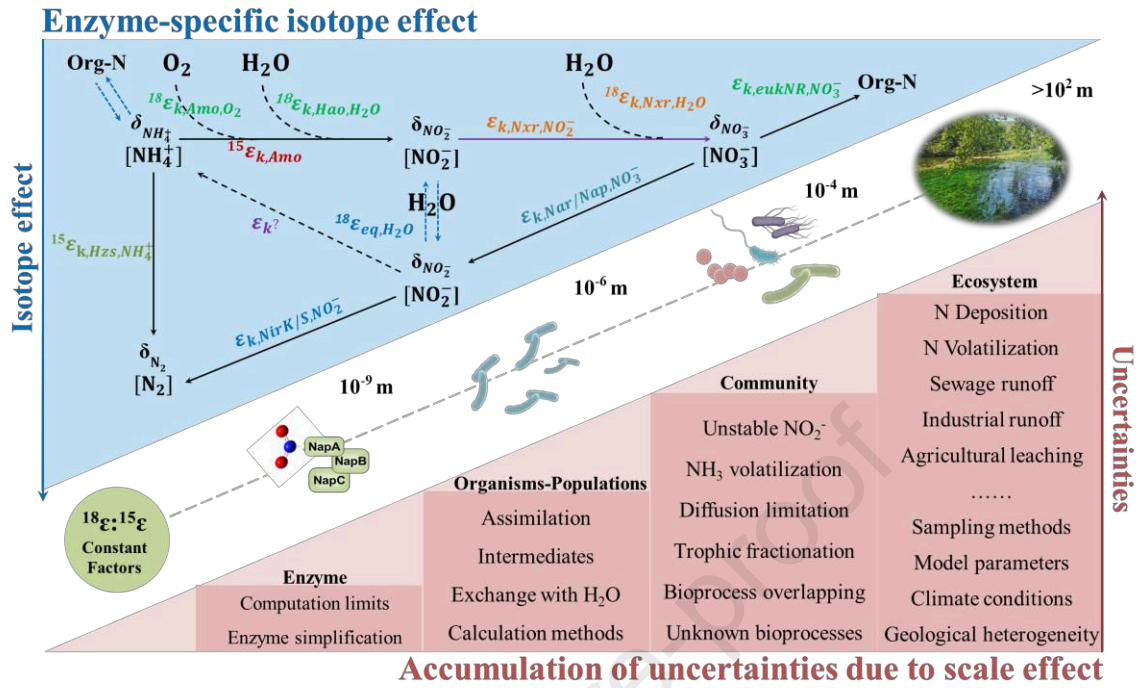
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CRedit authorship contribution statement

- Shuo Wang: Conceptualization; Methodology; Data curation; Writing–original draft.
- Shengjie Li, Zhuo Jiang, Zhengzhu Dang, Xianfang Zhu: Investigation.
- Tao Lyu, Wei Hu, Fu-jun Yue: Writing–review & editing.
- Guodong Ji: Conceptualization; Writing–review & editing; Project administration.



Unignorable Enzyme-specific Isotope Fractionation for Nitrate Source Identification in Aquatic Ecosystem

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Abstract

Nitrate contamination in aquatic systems is a widespread problem across the world. The isotopic composition ($\delta^{15}\text{N}$, $\delta^{18}\text{O}$) of nitrate and their isotope effect ($^{15}\epsilon$, $^{18}\epsilon$) can facilitate the identification of the source and transformation of nitrate. Although previous researches claimed the isotope fractionations may change the original $\delta^{15}\text{N}/\delta^{18}\text{O}$ values and further bias identification of nitrate sources, isotope effect was often ignored due to its complexity. To fill the gap between the understanding and application, it is crucial to develop a deep understanding of isotopic fractionation based on available evidence. In this regard, this study summarized the available methods to determine isotope effects, thereby systematically comparing the magnitude of isotope effects ($^{15}\epsilon$ and $^{18}\epsilon$) in nitrification, denitrification and anammox. We found that the enzymatic reaction plays the key role in isotope fractionations, which is significantly affected by the difference in the affinity, substrate channel properties and redox potential of active site. Due to the overlapping of microbial processes and accumulation of uncertainties, the significant isotope effects at small scales inevitably decrease in large-scale ecosystems. However, the proportionality of N and O isotope fractionation ($\delta^{18}\text{O}/\delta^{15}\text{N}$; $^{18}\epsilon/^{15}\epsilon$) associated with nitrate reduction generally follows enzyme-specific proportionalities (i.e., Nar, 0.95; Nap, 0.57; eukNR, 0.98) in aquatic ecosystems, providing enzyme-specific constant factors for the identification of nitrate transformation. With these results, this study finally discussed feasible source portioning methods when considering the isotope effect and aimed to improve the accuracy in nitrate source identification.

Keywords: Eutrophication control, nitrate source identification, enzyme-specific isotope effects, stable isotopes

1. Introduction

Anthropogenic activities have greatly increased inputs of bioavailable nitrogen into aquatic ecosystems, leading to unprecedented environmental degradation (Stein and Klotz, 2016; Kuypers et al., 2018; Zhang et al., 2020). Nitrate (NO_3^-) is the most dominant form and its concentration has risen significantly in freshwaters across the world, which poses serious threats (e.g., eutrophication) to aquatic ecosystems (Xuan et al., 2022). Moreover, high levels of nitrate can also increase the risk of certain diseases associated with drinking water (Lorette et al., 2022; Zaryab et al., 2022; Zhang et al., 2022). The main sources of nitrate pollution in waters are treated sewage effluent, industrial runoff, agricultural leaching (chemical fertilizer and manure) and atmospheric precipitation, in which the organic nitrogen and ammonia are transformed into nitrate through mineralization and nitrification processes. Although nitrate can be thoroughly removed through the processes of denitrification, the natural environment is not always in favour of such biogeochemical reactions. Therefore, accurately identifying the sources of nitrate in waters and correspondingly cutting down the input has become the key approach to help prevent environmental degradation.

Stable isotopes of nitrogen (^{14}N , ^{15}N) and oxygen (^{16}O , ^{17}O , ^{18}O), along with their fractionation characteristics leave detectable traces for nitrate identification, enabling the stable isotope technique to effectively quantify the source and transformation of nitrate (Granger and Wankel, 2016). The isotopic composition ($\delta^{15}\text{N}$, $\delta^{18}\text{O}$) of nitrate varies with different sources, which has been used as an indicator to identify the source of nitrate (Xue et al., 2009). The developed Bayesian stable isotope mixing models (e.g., MixSIAR) further contribute to the data analysis and the visualisation of outputs. Thereby increasing studies have combined dual isotopes and the Bayesian model to quantify the nitrate sources in the river (Ryu et al., 2021a), groundwater (Torres-Martínez et al., 2020), wetland (Hu et al., 2020), and precipitation (Shi et al., 2021). Additionally, as it is easier for lighter isotopes to break bonds during microbial nitrogen transformations, the molecules containing lighter isotopes react faster than those containing heavier isotopes, leading to a progressive enrichment of the heavy isotope in the substrate (Fry, 2006; Michener and Lajtha, 2007). Therefore, the microbial metabolic nitrogen transformation pathways can be traced based on specific isotope enrichment factors ($^{15}\epsilon$ and $^{18}\epsilon$).

However, the current implementation of the stable isotope analysis still faces the challenge of identifying accurate isotope fractionations (N and O) during nitrogen transformation processes. Although the isotopic composition ($\delta^{15}\text{N}$, $\delta^{18}\text{O}$) of nitrate from different sources have been well summarized in previous studies, the isotope fractionations may change the original $\delta^{15}\text{N}/\delta^{18}\text{O}$ values and further bias identification of nitrate sources (Fenech et al., 2012; Xue et al., 2009). Nevertheless, isotope fractionations are often ignored due to its complexity (Wong et al., 2014; Kang and Xu, 2016; Fang et al., 2012). The transformation of nitrate is influenced by the joint effects of anthropogenic activities and natural conditions in complex ecosystems with multiple nitrogen source. Although the available data of N isotope effects has been systematically summarized for various key ecosystem processes (Denk et al., 2017), the O

isotope effects has not been well discussed. Moreover, enzymes, rather than microbial processes, ultimately determine isotopic fractionation. However, the mechanism of enzyme-specific isotope fractionation has not been well reviewed. To fill the gap between the understanding and application, it is crucial to develop a deep understanding of isotopic fractionation underpinning mechanisms based on available evidence.

This study aims to summarise the evidence of isotope fractionations in nitrate source identification and propose the appropriate practices for nitrate source identification when considering isotopic effect. Firstly, the enzymatic mechanisms of isotope fractionation were reviewed. Secondly, as enzyme plays a key role in isotopic fractionation, the enzyme-specific isotope effects and culture-based basic data, as well as the constant factors at cross-scales from enzyme to ecosystem were summarized. Finally, this paper discusses the appropriate practices for nitrate source identification with the decrease of apparent isotopic effects in aquatic system. The summary of the isotopic fractionation associated with nitrate microbiological transformations will further promote the understanding of the nitrate source and transformation in aquatic ecosystems, ultimately achieving the goal of protecting the aquatic environment.

2. Theoretical calculation of isotope fractionations

The understanding of enzyme-specific isotope fractionation is crucial for the identification of nitrate sources in macroscale investigations (He et al., 2021). During the enzymatic transformation of nitrate, the substrate (S) is not transformed into products directly, but bound with an enzyme (E) to form a substrate-enzyme complex intermediate SE (Eq. 1). For example, in the reaction of nitrate reduction by periplasmic dissimilatory nitrate reductase (Nap), the substrate represents nitrate and the enzyme Nap combines with nitrate to form the substrate-enzyme complex. Then, the substrate-enzyme complex with high potential energy could further generate the final product (P).



The formation of the substrate-enzyme complex is reversible (Eq. 1), and the equilibrium fractionation during this process is accounted as the kinetic isotope effect (KIE). The traditional approach to calculating the KIE follows Eq. 2 (Mariotti et al., 1981).

$$KIE = \frac{k}{^hk} \quad (\text{Eq. 2})$$

where k and ^hk denote the reaction rate constants for the light and the heavy isotope-bearing molecules, respectively.

In the culture-based experiment, the available evidence of nitrate isotope fractionation is commonly summarised from the isotope ratios of either nitrogen or oxygen in substrate E and product P, i.e., $\alpha_{s/p}$. It is unified with KIE (Eq. 2) for the first order reaction (i.e., $\alpha_{s/p} = k/^hk$, See the details in SI). For the convenient comparability between isotopic values, the isotope fractionation effect can also be expressed as the isotope enrichment factor $\varepsilon_{s/p} = 10^3 (\alpha_{s/p} - 1)$.

As the reaction rate constants of different isotopes vary (Bigeleisen and Mayer, 1947; Urey, 1947; He et al., 2021), the formation of statistical thermodynamics is used to identify the equilibrium constants of light (Eq. 3) and heavy (Eq. 4) isotopes.

$$k = \frac{Q_{SE}}{Q_S \times Q_E} = \frac{S_S \times S_E}{S_{SE}} \times \frac{\prod_i^{3N_{SE}-7} (u_{i,SE} \times e^{-u_{i,SE}/2} \times \frac{1}{1-e^{-u_{i,SE}}})}{\prod_i^{3N_S-6} (u_{i,S} \times e^{-u_{i,S}/2} \times \frac{1}{1-e^{-u_{i,S}}}) \times \prod_i^{3N_E-6} (u_{i,E} \times e^{-u_{i,E}/2} \times \frac{1}{1-e^{-u_{i,E}}})} \quad (\text{Eq. 3})$$

$$^h k = \frac{Q_{SE}^*}{Q_S^* \times Q_E^*} = \frac{S_S^* \times S_E^*}{S_{SE}^*} \times \frac{\prod_i^{3N_{SE}^*-7} (u_{i,SE}^* \times e^{-u_{i,SE}^*/2} \times \frac{1}{1-e^{-u_{i,SE}^*}})}{\prod_i^{3N_S^*-6} (u_{i,S}^* \times e^{-u_{i,S}^*/2} \times \frac{1}{1-e^{-u_{i,S}^*}}) \times \prod_i^{3N_E^*-6} (u_{i,E}^* \times e^{-u_{i,E}^*/2} \times \frac{1}{1-e^{-u_{i,E}^*}})} \quad (\text{Eq. 4})$$

where Q_{SE} , Q_S and Q_E are partition functions of transition state SE, substrate S and enzyme E with the light isotope, while those with the superscript “*” indicate partition functions of heavy isotope substituted molecules. S_{SE} , S_S and S_E are symmetry numbers of transition state SE, substrate S and enzyme E, the change of which is negligible after the exchange of isotopes ($S \approx S^*$). N_S and N_{SE} are the total number of atoms; h denotes the Planck constant, ν_i denotes the i^{th} harmonic vibration frequency, k denotes the Boltzmann constant, and T denotes the temperature in K.

Due to the large molecular weight of the enzyme, the isotope fractionation for the enzyme itself is neglectable ($Q_E \approx Q_E^*$) (He et al., 2021). Therefore, the KIE can be obtained based on Eq. 5.

$$\text{KIE} = \frac{\prod_i^{3N_S-6} (u_{i,S}^* \times \frac{e^{-u_{i,S}^*/2}}{e^{-u_{i,S}/2}} \times \frac{1-e^{-u_{i,S}}}{1-e^{-u_{i,S}^*}})}{\prod_i^{3N_{SE}-7} (u_{i,SE}^* \times \frac{e^{-u_{i,SE}^*/2}}{e^{-u_{i,SE}/2}} \times \frac{1-e^{-u_{i,SE}}}{1-e^{-u_{i,SE}^*}})}; \quad u_i = \frac{h\nu_i}{kT} \quad (\text{Eq. 5})$$

where the numerator and denominator are reduced partition function ratios of substrate S (RPF_{R_S}) and transition state SE (RPF_{R_{SE}}) respectively. In the formula of RPF_R, only one parameter, the vibration frequency ν_i , is unknown. Therefore, the theoretical values of the isotope fractionation effect can be obtained by getting the vibration frequencies of substrate S and transition state SE.

The key to calculating the vibration frequencies (ν_i) of substrate S and transition state SE is to figure out the structure of the enzyme and its active site, as well as the process of bond formation or rupture. Due to current limitations of computational resources, it is not able to calculate the enzyme with a large molecular size. Therefore, the KIE calculation of an enzymatic reaction based on Density Functional Theory (DFT) and Gaussian software needs to be simplified to a cluster by cut-off method (Wolfsberg and Stern, 1964; Stern and Wolfsberg, 1966), where the active site is located at the centre of the cluster with close proximal surroundings. All geometry optimization and harmonic vibrational frequencies calculation for ground states can be performed in Gaussian software (Casciotti, 2009; Frisch et al., 2016). During nitrate reduction, the theoretical values of isotope fractionation for periplasmic dissimilatory nitrate reductase (Nap) are $^{15}\epsilon = 31.8 \pm 2.3\text{‰}$ and $^{18}\epsilon = 20.6 \pm 0.9\text{‰}$ (He et al., 2021), which is compared to the culture-based values of Nap ($^{15}\epsilon = 38.6 \pm 1.7\text{‰}$; $^{18}\epsilon = 19.30 \pm 0.85\text{‰}$) (Table 1). As the investigations in the structure, active site and bond formation/rupture mechanism of enzymes are inadequate, only during a few simple microbial processes in the

nitrogen cycle, e.g., nitrate reduction and nitrite oxidation, isotopic fractionations have been calculated by quantum chemical methods. Isotopic fractionations during microbial processes in the nitrogen cycle still require further study to better explore the microscopic mechanism.

Table 1 Data summary of nitrate isotopic data (‰) reported in previous studies (individual data can be found in Table S1-18, supplementary material).

		Enzyme		Organisms-Populations		
		N	O	N	O	H ₂ O
Nitrification	$\varepsilon_{k,Amo,AOB}$	-	-	27.0±7.1 ^a	21.0±1.1 ^b (Amo&Hao)	
	$\varepsilon_{k,Amo,AOA}$	-	-	24.5±7.9 ^c	-	
	$\varepsilon_{k,Nxr}$	-	-	-16.8±5.3 ^d	-6.1±3.2 ^d	15.1 ± 0.9 ^d
Denitrification	$\varepsilon_{k,Nap}$	31.8±2.3 ^e	20.6±0.9 ^e			
		38.6±1.7 ^f	19.3±0.9 ^f	22.3±6.1 ⁱ	12.0±2.6 ⁱ	-
	$\varepsilon_{k,Nar_based}$	28.0±2.0 ^g	27.1±1.5 ^g	21.2±5.2 ^j	19.1±4.8 ^j	
	$\varepsilon_{k,eukNR}$	26.9±1.32	26.5±2.5 ^h	11.6±3.4 ^k	11.6±3.8 ^k	-
	$\varepsilon_{k,NirK}$	-	-	21.7±1.8 ^l	2.1±2.2 ^l	-
	$\varepsilon_{k,NirS}$	-	-	8.4±2.1 ^m	6.0±3.1 ^m	-
Anammox	$\varepsilon_{k,Hzs}$	-	-	32.2±1.9 ^o	-	-
	$\varepsilon_{k,NirK}$	-	-	29.5±3.9 ^p	-	-
	$\varepsilon_{k,NirS}$	-	-	16.0±3.3 ^q	-	-
	$\varepsilon_{k,Nxr}$	-	-	-32.4±4.9 ^r	-2.2±1.1 ^s	

Note: a, Table S1, n=16; b, Table S2, n=7; c, Table S3, n=30; d, Table S4, n=13 (Delwiche and Steyn, 1970; Mariotti et al., 1981; Casciotti, 2009; Casciotti et al., 2003; Santoro and Casciotti, 2011; Buchwald and Casciotti, 2010; Casciotti et al., 2010; Buchwald et al., 2012; Mooshammer et al., 2020). e, Table S5, n=18, theoretical values; f, Table S6, n=2; g, Table S7, n=23; h, Table S8, n=11; i, Table S9, n=41; j, Table S10, n=47; k, Table S11, n=23; l, Table S12, n=12; m, Table S13, n=14 (Asamoto et al., 2021; Casciotti, 2009; Frey et al., 2014; Granger et al., 2008, 2006, 2004; Granger and Wankel, 2016; He et al., 2021; Karsh et al., 2012; Martin and Casciotti, 2016; Treibergs and Granger, 2017; Wunderlich et al., 2012). o, Table S14, n=14; p, Table S15, n=4; q, Table S16, n=14; r, Table S17, n=15; s, Table S18, n=10 (Brunner et al., 2013; Kobayashi et al., 2019; Kotajima et al., 2020).

3. Isotope fractionations of nitrate in microbial reactions

The experimental system boundaries could significantly affect the results obtained from the studies on isotope fractionations of nitrate, which include the following three scenarios.

1) Rayleigh fractionation in a closed system. The closed system is usually distinguished by the limited supply of substrate, and reactions progress sequentially and irreversibly fashion in an isolated system (Kendall and McDonnell, 1998), for example, closed-bottle batch cultures in the laboratory or sequencing batch reactor. As the accumulation of products has no impact on the reaction, $\alpha_{s/p} = R_s/R_{pi}$, where R_{pi} is the isotope ratio of the instantaneous product which appears in an infinitely short time at time t , and R_s the isotope ratio of the substrate at the same time (Fry, 2006). As the light isotopes react faster than the heavy isotopes, heavy isotopes enrich in the substrate ($\delta_s = \delta_{s,0} - \varepsilon_{s/p} \ln f$) and the light isotopes accumulate in the product ($\delta_{pi} = \delta_s - \varepsilon_{s/p}$), and the compositions of the instantaneous product and substrate differ by $\varepsilon_{s/p}$ at all times (Figure 1, black lines). In addition, at the end of the reaction, when

all the substrate is transformed into a product, the final δ values of the cumulative product (δ_p) are equal to that of the initial substrate ($\delta_{s,0}$).

2) Non-Rayleigh fractionation in an open system. The open system is usually distinguished by a continual supply of substrate, products and residual substrate (unreacted fraction f) flow out in a fixed proportion (Kendall and McDonnell, 1998), such as continuous cultures with balanced inputs and outputs or continuous flow reactor. For example, substrate nitrate (10 mg/L) is continually supplied into a reactor, and products (nitrite, 6 mg/L) and residual substrate (nitrate, 4 mg/L) flow out with the unreacted fraction $f = 0.4$. As the accumulation of products has an impact on the reaction, $\alpha_{s/p} = R_s/R_p$, where R_p and R_s are the ratios of the heavy isotope to the lighter isotope in cumulative product and substrate. Thus, we can obtain the δ values of the substrate (δ_s) and cumulative product (δ_p) according to the red lines in Figure 1.

3) Infinite system. When the substrate is infinite, f identically equal to 1, whether in a closed system or open system, we can get the relationship illustrated by blue lines. Examples are the incorporation of oxygen gas (O_2) and water (H_2O) during nitrification or nitrogen fixation, where the substrate can be regarded infinite and the reaction has no effect on its isotope composition, thus the isotope compositions of substrate and product remain stable as illustrated in the blue lines (Figure 1).

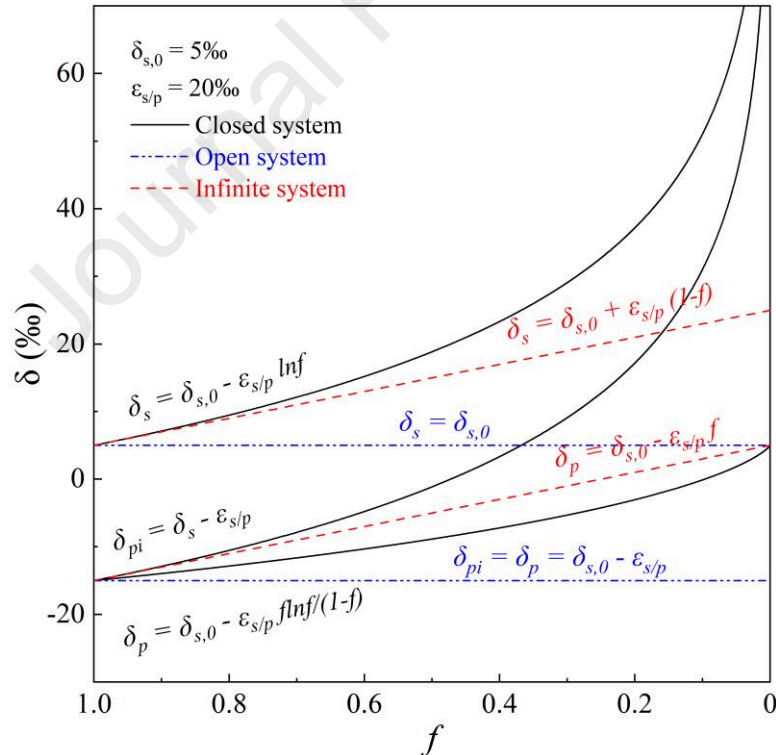


Figure 1. Relative changes in δ values of substrate (δ_s), instantaneous product (δ_{pi}) and cumulative product (δ_p) during kinetic fractionation processes in closed system (black lines), open system (red lines) and infinite system (blue lines). The x-axis f indicates the unreacted fraction of substrate. See the derivation process in SI.

198 **3.1 Nitrification**

Nitrification is one of the main sources of nitrate in aquatic ecosystems (Kuypers et al., 2018), which is a microbially-catalysed process whereby ammonium (NH_4^+) is oxidized to nitrite (NO_2^-) and then to nitrate (NO_3^-) by ammonium-oxidizing bacteria (AOB), ammonium-oxidizing archaea (AOA), and nitrite-oxidizing bacteria (NOB).

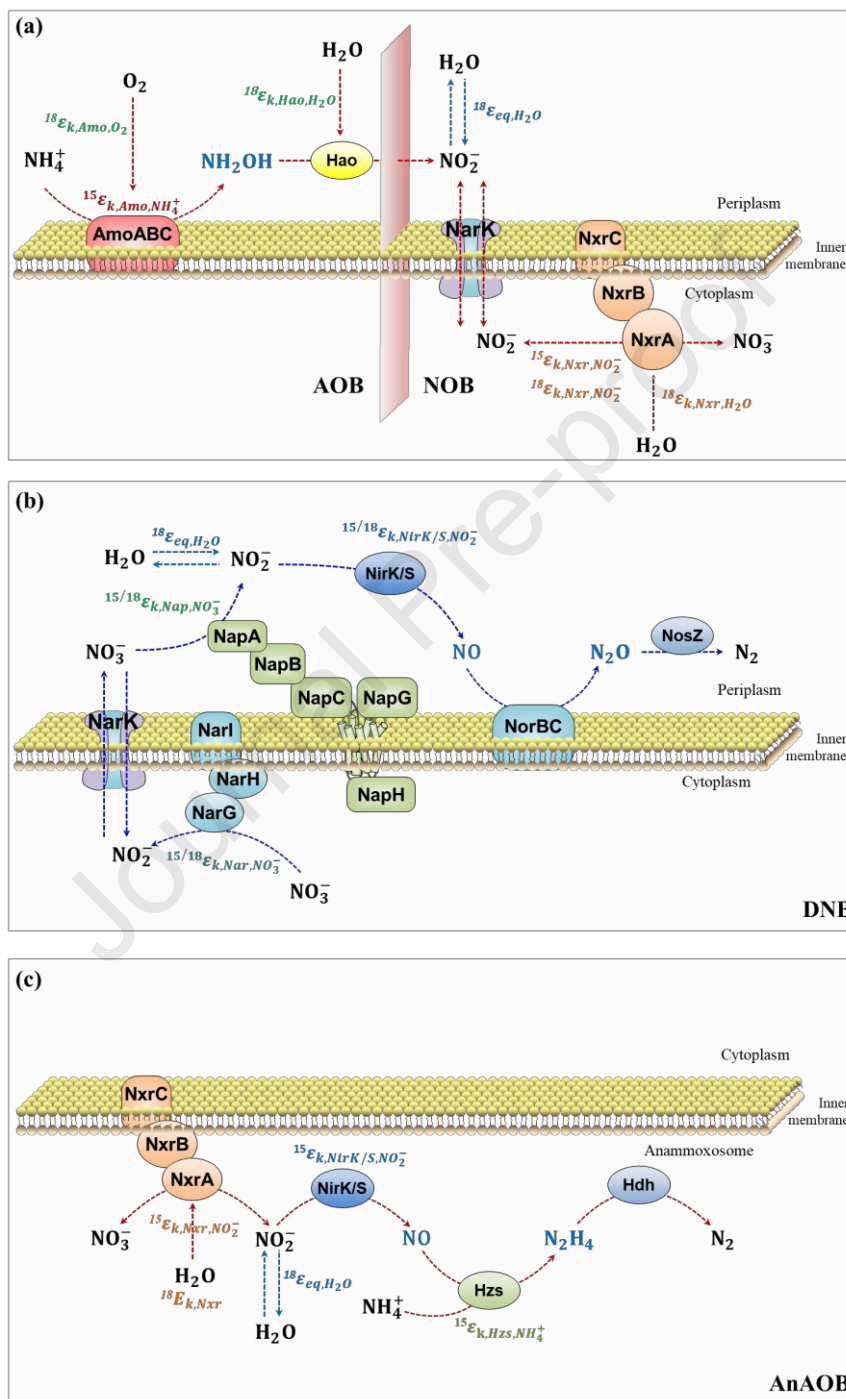


Figure 2. Schematic overview of processes and enzyme-specific isotope effects involved in (a) nitrification. Abbreviations: Amo, ammonia monooxygenase; Hao, hydroxylamine oxidoreductase; Nxr, nitrite oxidoreductase; Nar, membrane-bound nitrate reductase; AOB, ammonium-oxidizing bacteria; NOB, nitrite-oxidizing bacteria. (b) denitrification. Abbreviations: Nar, membrane-bound nitrate reductase; Nap,

periplasmic nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase; DNB, denitrifying bacteria. (c) anammox. Abbreviations: Nxr, nitrite oxidoreductase; Nir, nitrite reductase; Hzs, hydrazine synthase; Hdh, hydrazine dehydrogenase; AnAOB, anaerobic ammonium-oxidizing bacteria.

3.1.1 Ammonia oxidation

As illustrated in Figure 2a, ammonium is first oxidized by ammonia monooxygenase (Amo) to hydroxylamine, which is further oxidized by hydroxylamine oxidoreductase (Hao) to NO_2^- by AOB. The nitrogen isotope effects for Amo during ammonia oxidation are generally obtained in closed-bottle batch cultures (Figure 1, black lines). The nitrogen isotope enrichment factor $^{15}\epsilon_{k,\text{Amo,AOB}}$ ranges from 14.2‰ to 38.2‰ with an average of 27.0 ± 7.1 ‰ (Figure 3, Table 1). Compared with AOB, the $^{15}\epsilon_{k,\text{Amo,AOA}}$ has a wider range (from 10.0 to 40.1‰) with an average of 24.5 ± 7.9 ‰, but no significant difference is observed between $^{15}\epsilon_{k,\text{Amo,AOB}}$ and $^{15}\epsilon_{k,\text{Amo,AOA}}$ (Figure 3, Table 1). As the final isotopic values of the cumulative product (δ_p) are equal to that (δ_s) of the initial substrate (Figure 1, black lines), the microbiological process does not impart an isotope effect when there is no accumulation of intermediates. In the process of ammonium oxidation, hydroxylamine rarely accumulates, thus there is no isotope fractionation for Hao. Amo is regarded to play a vital role in isotope fractionation. It has been identified that AmoA amino acid sequences in four AOA strains, in which the similarities of gene sequences closely parallel the pattern of $^{15}\epsilon_{k,\text{Amo,AOA}}$ similarities, suggesting that genes affect isotopic fractionation by determining enzyme structure (Casciotti et al., 2003). Thus, we hypothesized that although the key enzyme like Hao in AOB has not been identified in AOA and AOB belong to different populations, they have similar isotope effects as they both have Amo enzyme.

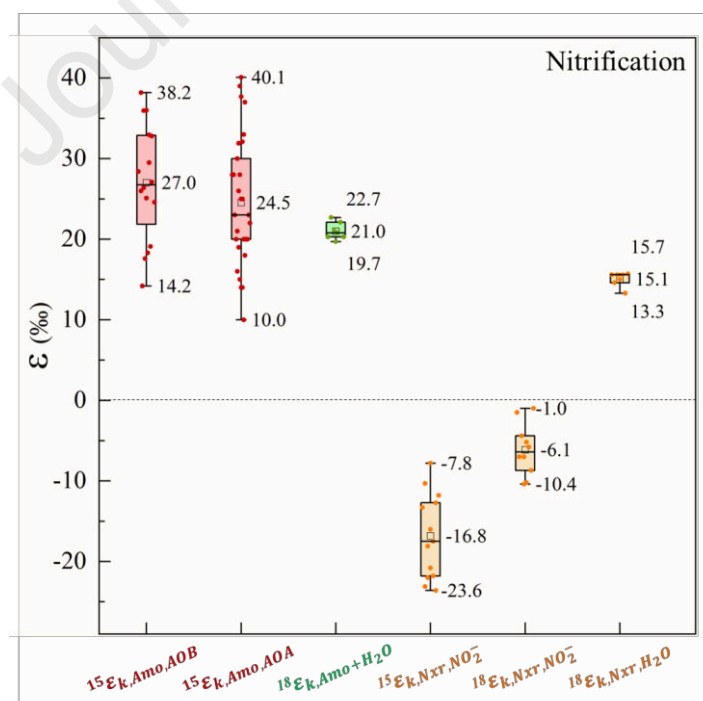


Figure 3. Boxplot of the enzyme-specific isotope effects for nitrification with data reported in the previous studies (Delwiche and Steyn, 1970; Mariotti et al., 1981; Casciotti, 2009; Casciotti et al., 2003; Santoro and

Casciotti, 2011; Buchwald and Casciotti, 2010; Casciotti et al., 2010; Buchwald et al., 2012; Mooshammer et al., 2020).

Compared to nitrogen, the isotope fractionation of oxygen is more complicated during nitrification. O_2 is incorporated during the oxidation of ammonia to hydroxylamine (NH_2OH), and H_2O is incorporated during the oxidation of both hydroxylamine to nitrite and nitrite to nitrate. Thus the ratio of 1:2 oxygen atoms from O_2 and H_2O can be used to interpret the oxygen isotopic composition of nitrate (Casciotti et al., 2010). However, a great basis was observed in the practical cultures and experiments. $\delta^{18}O-NO_3^-$ values are influenced not only by the oxygen isotopic composition of the H_2O and O_2 incorporated during nitrification, but also by the kinetic and equilibrium isotope effects during the reaction (Boshers et al., 2019).

Eq. 6 describes the oxygen isotope fractionation during ammonia oxidation, the first term of which represents the enzymatic incorporation of O_2 and H_2O during ammonia oxidation to nitrite. This part fractionation conforms to the conditions of infinite systems (Figure 1, blue lines) and the unreacted fraction f is identically equal to 1 during the incorporation of O_2 and H_2O . The second term represents the contribution of equilibrium isotope effects during the isotopic exchange of O atoms between nitrite and H_2O with the proportion of x_{eq} , which has a great uncertainty as a function of pH and temperature (Boshers et al., 2019). As hydroxylamine does not accumulate as intermediate products, the Hao has no isotopic effect on hydroxylamine (i.e., $^{15}\epsilon_{k,Hao,NH_2OH} = 0$; $^{18}\epsilon_{k,Hao,NH_2OH} = 0$). If Eq. 6 is arranged to group $\delta^{18}O_{H_2O}$ terms (Eq. 7), the intercept can be used to calculate the sum of the incorporation isotope effects for ammonia oxidation ($^{18}\epsilon_{k,Amo,O_2} + ^{18}\epsilon_{k,Hao,H_2O} = 21.0 \pm 1.1\%$) by using the exchange (x_{eq}) calculated from the slope (ranging from -0.05 ~ 1) and assuming $\delta^{18}O_{H_2O} = 24.2\%$ and $^{18}\epsilon_{eq,H_2O} = 12.5 \pm 0.5\%$ (Buchwald et al., 2012; Buchwald and Casciotti, 2010).

$$\delta^{18}O_{NO_2^-} = \left[\frac{1}{2} (\delta^{18}O_{O_2} - ^{18}\epsilon_{k,Amo,O_2}) + \frac{1}{2} (\delta^{18}O_{H_2O} - ^{18}\epsilon_{k,Hao,H_2O}) \right] (1-x_{eq}) + (\delta^{18}O_{H_2O} + ^{18}\epsilon_{eq,H_2O}) x_{eq} \quad (\text{Eq. 6})$$

$$\delta^{18}O_{NO_2^-} = \left(\frac{1}{2} + \frac{1}{2} x_{eq} \right) \delta^{18}O_{H_2O} + \frac{1}{2} (\delta^{18}O_{O_2} - ^{18}\epsilon_{k,Amo,O_2} - ^{18}\epsilon_{k,Hao,H_2O}) (1-x_{eq}) + ^{18}\epsilon_{eq,H_2O} x_{eq} \quad (\text{Eq. 7})$$

3.1.2 Nitrite oxidation

The nitrogen and oxygen isotope effects for nitrite oxidoreductase Nxr during nitrite oxidation can be calculated by the consumption of nitrite (Figure 1, black lines). The significant inverse isotope fractionation was observed in this process: $^{15}\epsilon_{k,Nxr,NO_2^-} = -16.8 \pm 5.3\%$ (from -23.6% to -7.8%), $^{18}\epsilon_{k,Nxr,NO_2^-} = -6.1 \pm 3.2\%$ (from -10.4% to -1.0%), breaking the rules that microbes preferentially use light isotopes. Some scientists believe that enzyme reversibility may lead to an apparent inverse isotope effect (Casciotti, 2009). If the reverse reaction has a larger isotope effect with sufficient mass flux than the forward reaction, it will lead to an apparent isotopic depletion of the original substrate pool, even if the forward and reverse reactions are normal. However, inverse isotope fractionation still occurs during nitrite

oxidation in the absence of a reversible reaction (<1%) (Casciotti, 2009). Therefore, the kinetic fractionation for nitrite oxidation is likely to be inverse at the enzyme level, which is also proved by the quantum chemical calculation. As only the bond formation exists during nitrite oxidation, if the isotopically substituted species is more stably bonded in the transition state, then molecules containing the heavy isotope may react more quickly. This process can lead to the accumulation of heavy isotopes during nitrate production, which distinguishes it from denitrification under natural conditions.

In addition, H₂O is incorporated during the oxidation of nitrite to nitrate, which is more complicated. Eq. 8 describes the newly-produced $\delta^{18}\text{O-NO}_3^-$, which includes 3 parts: (1) the isotopic exchange of O atoms between nitrite and H₂O; (2) Rayleigh fractionation induced by Nxr ($^{18}\epsilon_{k,Nxr,NO_2^-}$); (3) enzymatic incorporation of H₂O during nitrite oxidation ($^{18}\epsilon_{k,Nxr,H_2O}$).

$$\delta^{18}\text{O}_{NO_3^-,p_i} = \frac{2}{3} \{ [(1-x_{eq'}) \delta^{18}\text{O}_{NO_2^-} + x_{eq'} (\delta^{18}\text{O}_{H_2O} + ^{18}\epsilon_{eq,H_2O})] - ^{18}\epsilon_{k,Nxr,NO_2^-} \} + \frac{1}{3} (\delta^{18}\text{O}_{H_2O} - ^{18}\epsilon_{k,Nxr,H_2O}) \quad (\text{Eq. 8})$$

As the exchange of oxygen atoms between nitrite and H₂O during nitrite oxidation has been found to have a negligible effect on $\delta^{18}\text{O}_{NO_3^-}$ (Buchwald and Casciotti, 2010; Buchwald et al., 2012), $x_{eq'} \approx 0$, thus:

$$\delta^{18}\text{O}_{NO_3^-,p_i} = \frac{2}{3} (\delta^{18}\text{O}_{NO_2^-} - ^{18}\epsilon_{k,Nxr,NO_2^-}) + \frac{1}{3} (\delta^{18}\text{O}_{H_2O} - ^{18}\epsilon_{k,Nxr,H_2O}) \quad (\text{Eq. 9})$$

where $^{18}\epsilon_{k,Nxr,NO_2^-}$ belongs to Rayleigh fractionation (Figure 1, black lines) and $^{18}\epsilon_{k,Nxr,H_2O}$ belongs to the kinetic isotope effect in the infinite system. As the isotope value of an instantaneous product $\delta^{18}\text{O}_{NO_3^-,p_i}$ cannot be determined, Eq. 9 cannot be calculated directly. Considering that nitrite tend to be oxidized completely, we just focus on the endpoint of the reaction, when $^{18}\epsilon_{k,Nxr,NO_2^-} = 0$, nor need we consider the inverse kinetic isotope effect for NO_2^- conversion to NO_3^- . Thus Eq. 10 describes the $\delta^{18}\text{O-NO}_3^-$ when nitrite oxidation goes to completion:

$$\delta^{18}\text{O}_{NO_3^-,final} = \frac{2}{3} \delta^{18}\text{O}_{NO_2^-,initial} + \frac{1}{3} (\delta^{18}\text{O}_{H_2O} - ^{18}\epsilon_{k,Nxr,H_2O}) \quad (\text{Eq. 10})$$

For now, the intercept can be used to calculate the incorporation isotope effects for Nxr ($^{18}\epsilon_{k,Nxr,H_2O} = 15.1 \pm 0.9\text{‰}$) during nitrite oxidation (Buchwald and Casciotti, 2010).

3.2 Denitrification

Denitrification is the dominant nitrate loss process in nature, in which nitrate is converted to dinitrogen in the sequence of NO_2^- , NO, N₂O and N₂ (Figure 2b). Isotopic fractionation in denitrification will directly change the characteristic isotopic values of nitrate, and thus affect the source partitioning (Yu et al., 2020; Jiang et al., 2021; Ryu et al., 2021b; Shi et al., 2021).

3.2.1 Nitrate reduction

There are four major enzymes during nitrate reduction, including membrane-bound (Nar) and periplasmic (Nap) dissimilatory nitrate reductases in prokaryotes, eukaryotic (eukNR) and

prokaryotic (Nas) assimilatory nitrate reductases (Zhang et al., 2020). Many organisms contain both *nap* and *nar* gene clusters, and express both of them under anaerobic conditions while only *nap* gene clusters under aerobic conditions (Granger et al., 2008; Kuypers et al., 2018). The statistical data (Table S7, Table S10) demonstrate that Nar reductases usually play a major role when microorganisms contain both Nar and Nap enzymes from the perspective of isotope fractionation. Thus, the organisms with both enzymes and those harbours only Nar are both classified as Nar-based denitrifiers, whose nitrogen and oxygen isotope enrichment factors are $21.2 \pm 5.2\text{‰}$ and $19.1 \pm 4.8\text{‰}$ (Table 1). Nap reductase has been implicated in both aerobic and anaerobic denitrification, DNRA, but it may be inhibited by oxygen in some cases (Frey et al., 2014; Asamoto et al., 2021). The nitrogen and oxygen isotope enrichment factors for Nap in microbial cultures are $22.3 \pm 6.1\text{‰}$ and $12.1 \pm 2.6\text{‰}$, respectively (Table 1). In addition, in surface water with high algae density, the assimilatory reduction of nitrate will also significantly affect nitrate isotope values. The enrichment factors for eukNR in microbial cultures are $^{15}\epsilon = 11.6 \pm 3.4\text{‰}$ and $^{18}\epsilon = 11.6 \pm 3.8\text{‰}$, respectively (Table 1), significantly lower than those of Nar and Nap.

During nitrate reduction, nitrogen and oxygen isotope fractionations differ with different types of enzymes contained by microorganisms. The oxygen and nitrogen isotope fractionations also cover a broad range due to the population differences, but the proportionality of oxygen and nitrogen isotope fractionation ($^{18}\epsilon/^{15}\epsilon$) remains stable (Figure 4). Nar-based denitrifiers follows a proportionality of 0.95 ± 0.03 , the same as that of eukNR (0.98 ± 0.03), but they are significantly higher than that of Nap (0.57 ± 0.04). This invariant coupling of oxygen and nitrogen isotopic enrichments has been shown to originate from fractionation during enzymatic bond-breakage, which remains stable at the level of enzyme, individual and population, providing a cross-scale constant factor for identification of the nitrate reduction process. The Nar active site, Mo centre is coordinated by an aspartate residue, whose substrate channel is negatively charged that may impact the rate of nitrate binding (Jormakka et al., 2004). Additionally, the general process for nitrate binding allows nitrate to bind either Mo(V) or Mo(IV), and this reduction step depends on internal electron transfer (Anderson et al., 2001; Frey et al., 2014; Marangon et al., 2012). In contrast, the Nap reductase is coordinated by a cysteine, its substrate channel is lined with positively charged amino acid residues that guide nitrate to the active site (Najmudin et al., 2008; Coelho and Romão, 2015). Thus Nap generally has a higher affinity for nitrate than Nar (Arnoux et al., 2003; Pinho et al., 2005; Gates et al., 2008). Furthermore, nitrate binds the Mo centre only in the reduced state, Mo(IV), and reduces the nitrate immediately (Sparacino-Watkins et al., 2014; Coelho and Romão, 2015). Therefore, the different affinity, electrical properties of the substrate channel and redox potential of the Mo center may be key in understanding the differences in $^{18}\epsilon / ^{15}\epsilon$ proportionality.

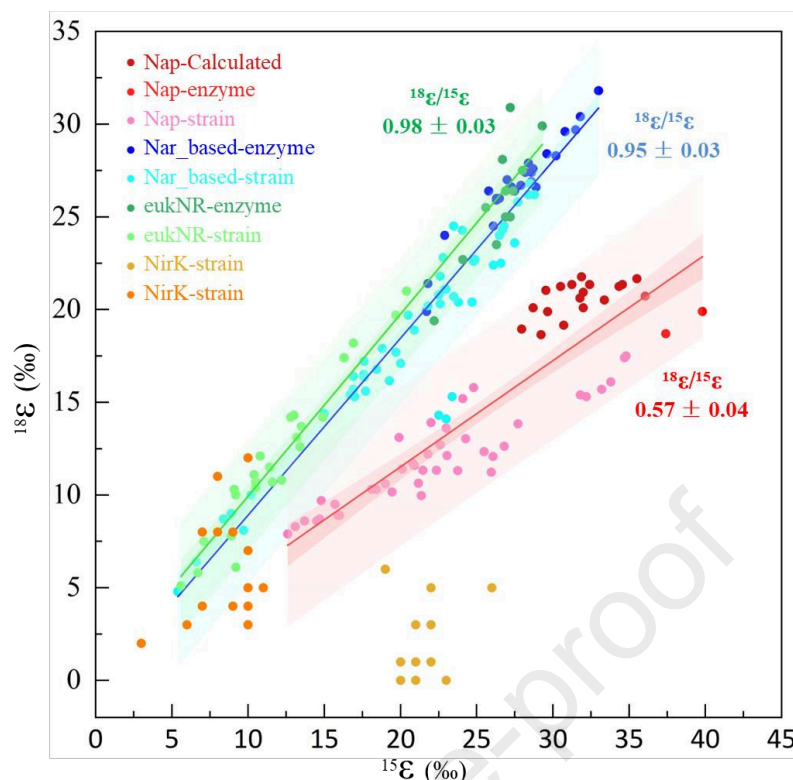


Figure 4. Compilation of enzyme-specific nitrate isotopic data during denitrification reported in the previous studies (Asamoto et al., 2021; Casciotti, 2009; Frey et al., 2014; Granger et al., 2008, 2006, 2004; Granger and Wankel, 2016; He et al., 2021; Karsh et al., 2012; Martin and Casciotti, 2016; Treibergs and Granger, 2017; Wunderlich et al., 2012).

3.2.2 Nitrite reduction

During the further reduction of nitrite, there are two distinct families of nitrite reductase (Nir) enzymes, which are mutually exclusive in any given strain (Coyne et al., 1989). One is a copper-containing enzyme (Cu-Nir) encoded by the *nirK* gene and another is a cytochrome cd₁-containing enzyme (Fe-Nir) encoded by the *nirS* gene. The nitrogen isotope enrichment factors in enzyme-catalysed reactions of NirK ($^{15}\epsilon_{k,NirK} = 21.7 \pm 1.8\text{‰}$) are almost 2~3 times that of NirS ($^{15}\epsilon_{k,NirS} = 8.4 \pm 2.1\text{‰}$). In contrast, NirK exhibits little oxygen isotope effect ($^{18}\epsilon_{k,NirK} = 2.1 \pm 2.2\text{‰}$), while NirS has a larger value of $^{18}\epsilon_{k,NirS} = 6.0 \pm 3.1\text{‰}$ (Martin and Casciotti, 2016). The difference between the isotope effects may be due to the different catalytic steps in how the enzymes bind nitrite. For the Fe-Nir, the Fe in the active site binds to the N of NO_2^- directly and then one of the N-O bonds is cleaved randomly (Murphy et al., 1997). While the Cu-Nir binds to both O atoms of NO_2^- and the N-O bond on the side closest to the Asp98 residue is cleaved (Fülöp et al., 1995). The equivalence in O atom positions for the Fe-Nir may offer more opportunity for O atom fractionation to occur during bond breakage, leading to a larger oxygen isotope effect (Martin and Casciotti, 2016). However, there is still no reliable explanation for nitrogen fractionation at present.

3.3 Anammox

In addition, anaerobic ammonium oxidation (anammox) by anaerobic AOB (AnAOB) can also significantly affect the isotopic characteristics of nitrate under certain conditions. NO_2^- is first reduced by NirK or NirS to NO, which subsequently undergoes comproportionation together with NH_4^+ into hydrazine under the catalysis of hydrazine synthase (Hzs) enzyme. Finally, hydrazine is oxidized to dinitrogen gas by hydrazine dehydrogenase (Hdh) (Kuypers et al., 2018). In addition, this process is also accompanied by nitrite oxidation catalysed by Nxr (Figure 2c). All AnAOB species have evolved the anammoxasome, which is made up of high-density lipids and can act as a barrier to diffusion thus isolating the toxic intermediates (i.e., NO, N_2H_4) of the anammox reaction within the anammoxasome (Strous et al., 2006). Therefore, as the intermediate products of anammox, NO and N_2H_4 will not accumulate and the enzymes catalysing the oxidation of NO and N_2H_4 do not undergo isotope fractionation, i.e., $^{15}\epsilon_{k,\text{Hzs},\text{NO}}$ and $^{15}\epsilon_{k,\text{Hdh},\text{N}_2\text{H}_4}$ are equal to 0.

As a multiple product reaction, the fractionation model in an open system is more suitable to calculate the isotope effect during anammox (Figure 1, red lines). As illustrated in Figure 5, NH_4^+ and NO_2^- are continually supplied, products and residual substrate flow out in a fixed proportion. As NH_4^+ flow out with unreacted fraction $f = C_{\text{NH}_4^+}/C_{\text{NH}_4^+,0}$, we can get $^{15}\epsilon_{k,\text{Hzs}}$ in the open system according to Eq. 11.

$$\delta^{15}\text{N}_{\text{NH}_4^+} = \delta^{15}\text{N}_{\text{NH}_4^+,0} + ^{15}\epsilon_{k,\text{Hzs}}(1-f) \quad (\text{Eq. 11})$$

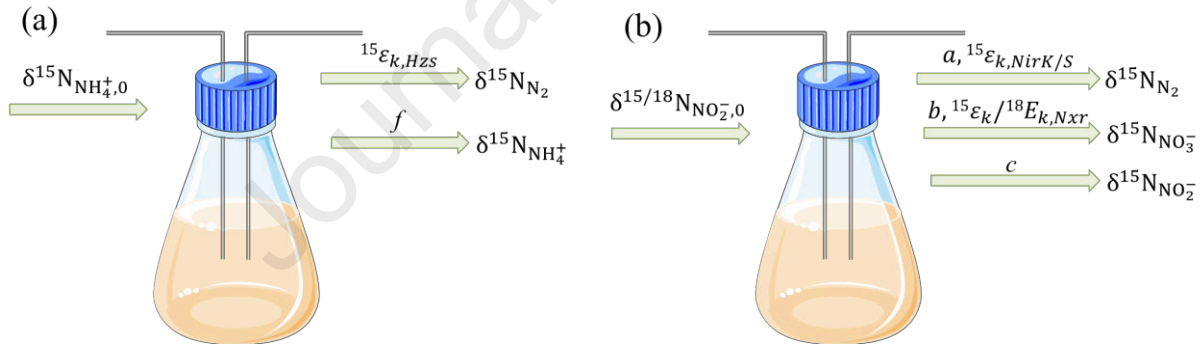


Figure 5. Diagram of a steady-state open system model. NH_4^+ and NO_2^- are continually supplied in an open system, (a) a product (N_2) is formed and residual substrate (NH_4^+) flows out in a fixed proportion (f); (b) two products (N_2 and NO_3^-) and unused substrate (NH_4^+) flow out in the fixed proportion of a , b and c .

The transformation of nitrite is more complicated, that is, the products N_2 and NO_3^- , along with the unreacted NO_2^- flow out in a proportion of a , b and c , where $a = (C_{\text{NH}_4^+,0} - C_{\text{NH}_4^+})/C_{\text{NO}_2^-,0}$; $c = C_{\text{NO}_2^-}/C_{\text{NO}_2^-,0}$; $a + b + c = 1$. In the open system:

$$\delta^{15}\text{N}_{\text{N}_2} = \delta^{15}\text{N}_{\text{NO}_2^-} - ^{15}\epsilon_{k,\text{nirS/K}} \quad (\text{Eq. 12})$$

$$\delta^{15}\text{N}_{\text{NO}_3^-} = \delta^{15}\text{N}_{\text{NO}_2^-} - ^{15}\epsilon_{k,\text{nxr}} \quad (\text{Eq. 13})$$

Thus:

$$\delta^{15}\text{N}_{\text{NO}_2^-,0} = a\delta^{15}\text{N}_{\text{N}_2} + b\delta^{15}\text{N}_{\text{NO}_3^-} + c\delta^{15}\text{N}_{\text{NO}_2^-}$$

$$\begin{aligned}
&= a(\delta^{15}\text{N}_{\text{NO}_2^-} - {}^{15}\epsilon_{\text{k,nirS/K}}) + b(\delta^{15}\text{N}_{\text{NO}_2^-} - {}^{15}\epsilon_{\text{k,nxr}}) + c\delta^{15}\text{N}_{\text{NO}_2^-} \\
&= \delta^{15}\text{N}_{\text{NO}_2^-} - a{}^{15}\epsilon_{\text{k,nirS/K}} - b{}^{15}\epsilon_{\text{k,nxr}}
\end{aligned} \tag{Eq. 14}$$

As ${}^{15}\epsilon_{\text{k,nxr}} = \delta^{15}\text{N}_{\text{NO}_2^-} - \delta^{15}\text{N}_{\text{NO}_3^-}$, $b = 1 - a - c$; We got:

$${}^{15}\epsilon_{\text{k,nirS/K}} = (\delta^{15}\text{N}_{\text{NO}_2^-} - \delta^{15}\text{N}_{\text{NO}_2^-,0} - b{}^{15}\epsilon_{\text{k,nxr}})/a \tag{Eq. 15}$$

For the oxygen isotope fractionation in anammox:

$$\begin{aligned}
\delta^{18}\text{O}_{\text{NO}_3^-} &= \frac{2}{3}(\delta^{18}\text{O}_{\text{NO}_2^-} - {}^{18}\epsilon_{\text{k,Nxr,NO}_2^-}) + \frac{1}{3}(\delta^{18}\text{O}_{\text{H}_2\text{O}} - {}^{18}\epsilon_{\text{k,Nxr,H}_2\text{O}}) \\
&= \frac{2}{3}\delta^{18}\text{O}_{\text{NO}_2^-} + \frac{1}{3}\delta^{18}\text{O}_{\text{H}_2\text{O}} - \left(\frac{2}{3}{}^{18}\epsilon_{\text{k,Nxr,NO}_2^-} + \frac{1}{3}{}^{18}\epsilon_{\text{k,Nxr,H}_2\text{O}}\right) \\
&= \frac{2}{3}\delta^{18}\text{O}_{\text{NO}_2^-} + \frac{1}{3}\delta^{18}\text{O}_{\text{H}_2\text{O}} - {}^{18}E_{\text{k,Nxr}}
\end{aligned} \tag{Eq. 16}$$

Thus:

$${}^{18}E_{\text{k,Nxr}} = \frac{2}{3}\delta^{18}\text{O}_{\text{NO}_2^-} + \frac{1}{3}\delta^{18}\text{O}_{\text{H}_2\text{O}} - \delta^{18}\text{O}_{\text{NO}_3^-} \tag{Eq. 17}$$

which is denoted as a combined oxygen isotope effect (Kobayashi et al., 2019; Kotajima et al., 2020).

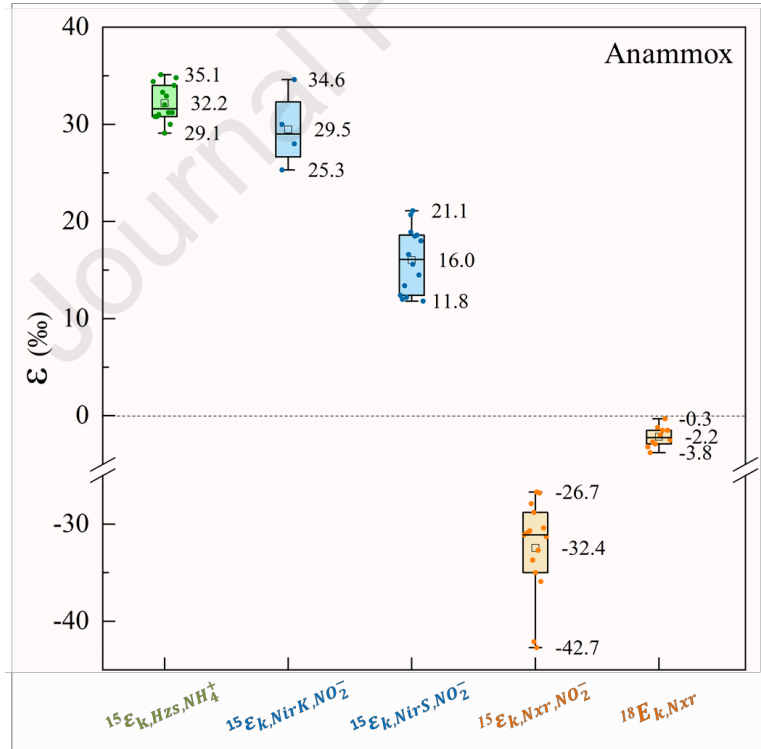


Figure 6. Boxplot of the isotope effects for anammox with data reported in the previous studies (Brunner et al., 2013; Kobayashi et al., 2019; Kotajima et al., 2020).

For the nitrogen isotope fractionation (Figure 6, Table 1), ${}^{15}\epsilon_{\text{k,Hzs}} = 32.2 \pm 2.9$ ‰, exhibiting significant isotope effects. In the process of nitrite reduction, ${}^{15}\epsilon_{\text{k,NirK}} = 29.5 \pm 3.9$ ‰, ${}^{15}\epsilon_{\text{k,NirS}} = 16.0 \pm 3.3$ ‰, which are consistent with the difference between ${}^{15}\epsilon_{\text{k,NirK}}$ and

$^{15}\epsilon_{k,NirS}$ during denitrification. In addition, the isotope fractionation trend is consistent with that of nitrification, exhibiting the inverse isotope fractionation ($^{15}\epsilon_{k,Nxr} = -32.4 \pm 4.9\text{‰}$, $^{18}\epsilon_{k,Nxr} = -2.2 \pm 1.1\text{‰}$). At the same time, the bacteria can perform DNRA, which is subsequently combined with NO_2^- to produce N_2 . This process cannot be determined for now because of the difficulty of the enrichment of DNRA.

4. Uncertainties and Appropriate practices for nitrate source identification

With the expansion of the research level, the accumulation of uncertainties leads to the decrease of apparent isotope fractionation effects, exhibiting an obvious gap between field-derived and culture-based isotopic signatures (Figure 7). As enzyme plays a key role in isotopic fractionation, the direct isotopic effect can be observed through enzymatic assays, which has no significant difference with the theoretical values (Table 1). However, Nar/Nap/eukNR fractionation effect (i.e., $^{15}\epsilon$ and $^{18}\epsilon$) significantly decreased from the enzyme scale to the organisms-populations scale (Figure 7). At this scale, nitrate transport, microbial assimilation, intermediates generation and the calculation methods could lead to inevitable deviations in the estimation of isotope effects (Denk et al., 2017). In aquatic ecosystems, each microbial process interacts, and it is usually the microbial community rather than the single microbial process that influences the nitrogen conversion and the isotope fractionation. The overlapping from biological processes such as nitrification, denitrification, anammox and some unknown biological processes further reduces the apparent fractionation effects (Figure 7, Figure 8). The phytoplankton assimilation, ammonia and nitrous acid volatilization, chemical decomposition and oxygen exchange between nitrite and water further increases the uncertainty of isotope fractionation (Heil et al., 2016; Knöller et al., 2011). Furthermore, these microbial process and chemical processes are all affected by environmental factors, such as pH value and temperature. As a result, the fractionation effect based on nitrate reduction is much smaller in natural waters, and sometimes even cannot be detected (Hu et al., 2020; Jiang et al., 2021; Lennon and Houlton, 2017; Li et al., 2019; Wu et al., 2019; Xia et al., 2017; Yu et al., 2020). Therefore, accurate interpretation strongly hinges on a deep understanding of the isotopic composition of nitrate sources and isotopic fractionation associated with nitrate biological transformations (Granger and Wankel, 2016). Appropriate practices for nitrate source identification without ignoring isotopic effects are discussed below.

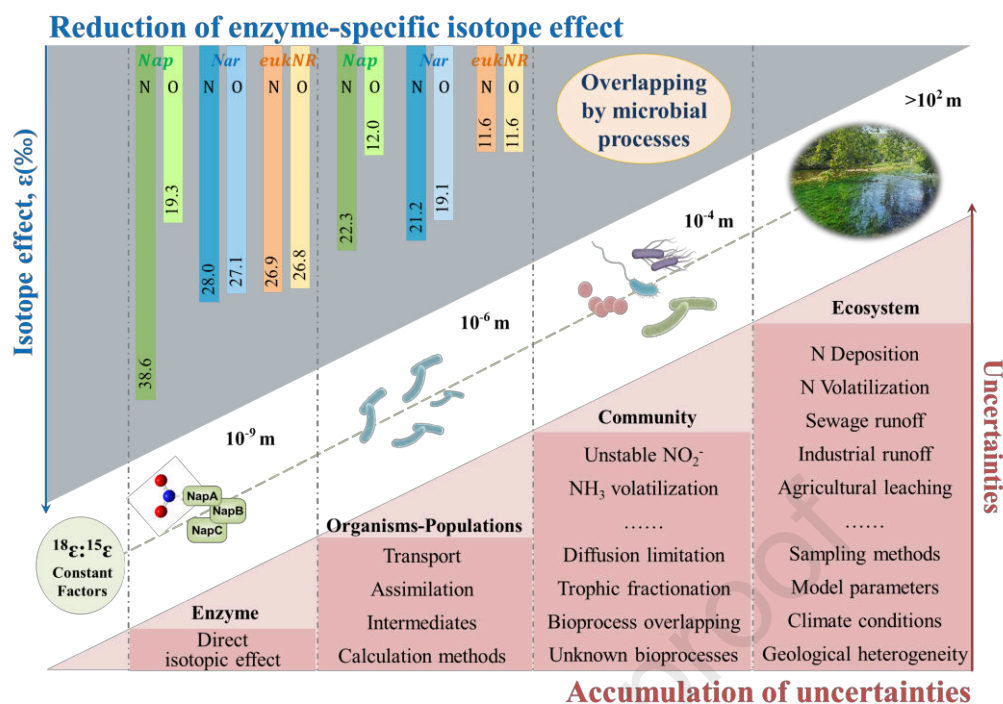


Figure 7. Reduction of enzymes-specific isotope effect and accumulation of uncertainties

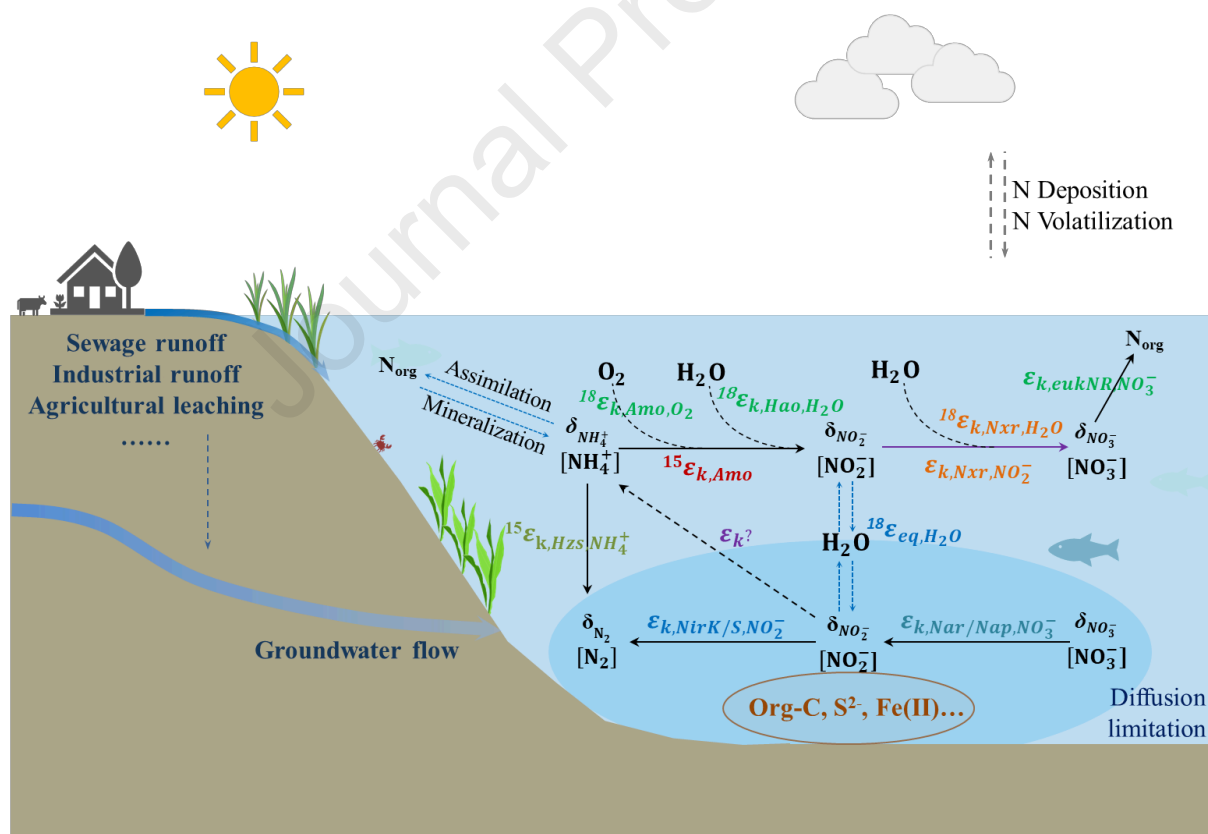


Figure 8. N transformations and associated enzyme-specific isotope effects at community-ecosystem scale. See the text and Table 1 for details.

4.1 Identification of nitrate generation and reduction

Significant isotopic fractionation was observed during nitrate generation and reduction. Denitrification processes can cause linear increases of $\delta^{15}\text{N}-\text{NO}_3^-$ and $\delta^{18}\text{O}-\text{NO}_3^-$ as the logarithm of the residual nitrate fraction decreases, while nitrification leads to linear increases of $\delta^{15}\text{N}-\text{NO}_3^-$ and $\delta^{18}\text{O}-\text{NO}_3^-$ as nitrate logarithmically increases. Therefore, apparent higher enrichment factors in natural water can indicate the nitrate reduction process (Wu et al., 2019; Li et al., 2019; Hu et al., 2020), and the apparent enrichment factor less than 0 can indicate the nitrification process (Zhang et al., 2022). However, the in-situ nitrogen and oxygen isotope enrichment factors based on nitrate were mostly lower than 5‰ due to the overlapping of microbial processes and accumulation of uncertainties, and even decreased to 0 in some cases. Although $^{18}\epsilon$ and $^{15}\epsilon$ significantly decreased with the increase in the scale from enzyme to ecosystem, the $^{18}\epsilon/^{15}\epsilon$ proportionality was consistent and provided a cross-scale constant factor for nitrate source identification (Figure 7). The positive correlation between oxygen and nitrogen isotopes of nitrate can serve as a denitrification diagnostic, while no positive correlation or negative pattern may be explained as a mixing of different pollution sources (Liu et al., 2006; Kaushal et al., 2011; Kang and Xu, 2016).

As mentioned above, $^{18}\epsilon/^{15}\epsilon$ was significantly different between Nar (0.95 ± 0.03) and Nap (0.57 ± 0.04) (Fig. 5). The proportionality of N and O isotope fractionation ($\delta^{18}\text{O}/\delta^{15}\text{N}$; $^{18}\epsilon/^{15}\epsilon$) associated with nitrate reduction in marine ecosystems generally follows a proportionality of ~ 1.0 (Granger et al., 2008; Asamoto et al., 2021). The large-scale anaerobic regions, like oxygen minimum zones (OMZs) and anoxic sediments in marine ecosystems provides the necessary redox conditions for Nar enzyme catalysis, thus Nar is probably the dominant enzyme in the dissimilation reduction of nitrate in marine ecosystems. In addition, the sulfide-driven autotrophic denitrification is the dominant N-loss pathway in some marine areas, where microbes only contain the Nap enzyme and can be applied to distinguish autotrophic and heterotrophic denitrification (Frey et al., 2014). In contrast to ocean environments, nitrate N and O isotope fractionations observed for denitrification in rivers and lakes are associated with a fractionation ratio of around 0.6 (Granger et al., 2008; Asamoto et al., 2021). In freshwaters, the water is basically aerobic in shallow rivers and lakes, and the anaerobic region at the bottom is often disturbed as well, thus Nar enzymes were often inhibited by the presence of O_2 above suboxic concentrations ($10 \mu\text{M}$) (Spiro and Guest, 1990; Moir and Wood, 2001; Wood et al., 2001), but the expression of Nap enzymes was promoted. In addition, the highly reduced carbon sources in freshwaters can also promote the expression of Nap reductases (Sears et al., 2000; Ellington et al., 2002; Knöller et al., 2011). Therefore, Nap reductases are probably the dominant enzymes during denitrification in freshwaters. In addition, $^{18}\epsilon/^{15}\epsilon = 0.98 \pm 0.03$ during eukNR-dominant assimilatory nitrate reduction can be used to determine the occurrence of assimilatory nitrate reduction in the surface aerobic zone (Chen et al., 2020; Wu et al., 2019). It is worth noting that the proportionality of N and O isotope fractionation will shift when the nitrate transformation in water is not dominated by reduction, and nitrification will lead to the increase of the $^{18}\epsilon/^{15}\epsilon$ ratio (Granger and Wankel, 2016; Wu et al., 2019).

4.2 Nitrate source partitioning

In nitrate source partitioning, the source of nitrate can be identified by collecting the characteristic isotopic values ($\delta^{15}\text{N}$, $\delta^{18}\text{O}$) of nitrate produced in nitrification or derived from major pollutants (atmospheric precipitation, sewage runoff, industrial runoff and agricultural leaching). However, the overlapping isotope compositions of nitrate sources will be further expanded by the isotope fractionations, leading to more uncertainties in source apportionment. Thus the consideration of isotope fractionations is strongly recommended for nitrate source apportionment in recent studies (Yu et al., 2020; Jiang et al., 2021; Ryu et al., 2021b). We have summarized the recent typical works in Table S19.

In the waters with high quality, such as rivers, shallow lakes or shallow groundwater, the nitrate reduction was not significant due to the high level of dissolved oxygen (Kang et al., 2022; Li et al., 2022; Ryu et al., 2021a), whereas nitrification is probably the main process of nitrate origination (Ryu et al., 2021a; Xuan et al., 2020). In this condition, the error caused by denitrification fractionation can be ignored, and the neglect of isotope fractionation in the nitrification process becomes the main source of uncertainties. Most research works adopt the nitrogen isotope of ammonia nitrogen and the direct combination of oxygen isotope in the atmosphere and in-situ water ($\delta^{18}\text{O}_{\text{NO}_3^-} = \frac{2}{3} \delta^{18}\text{O}_{\text{H}_2\text{O}} + \frac{1}{3} \delta^{18}\text{O}_{\text{O}_2}$) as the characteristic isotope values without considering the isotope effects in this process (Kang and Xu, 2016; Yu et al., 2020; Ryu et al., 2021b). Considering that the nitrogen isotope ratio of the accumulated product nitrate is close to that of the initial substrate when ammonia is completely oxidized to nitrate (Figure 1, black lines). It is reasonable to use the nitrogen isotope of ammonia nitrogen as the tracing characteristic isotopic value of nitrate. As for the oxygen isotope, although ammonia and nitrite are completely oxidized, isotopic fractionation still has a significant effect on it. The errors mainly derive from kinetic fractionation during H_2O and O_2 incorporation and the exchange between nitrite and water (Eq. 7). The basic data in microbial cultures can be used as a reference: $^{18}\epsilon_{k, \text{Amo}, \text{O}_2} + ^{18}\epsilon_{k, \text{Hao}, \text{H}_2\text{O}} = 21.0 \pm 1.1\text{‰}$ during the incorporation of H_2O and O_2 ; and $^{18}\epsilon_{k, \text{Nxr}, \text{H}_2\text{O}} = 15.1 \pm 0.9\text{‰}$ during the incorporation of H_2O to nitrite (Table 1), which has been applied in the previous studies (Jiang et al., 2021). Additionally, the oxygen exchange between nitrite and water is more complicated, and the isotopic exchange ratio of O atoms between nitrite and H_2O varies greatly and can even reach 100% under some conditions (Buchwald and Casciotti, 2010; Buchwald et al., 2012). Some research works added 5‰ uncertainty from possible kinetic and equilibrium fractionation during O incorporation (Buchwald and Casciotti, 2013; Wells et al., 2016).

For the waters with obvious nitrate reduction, errors are inevitable when using the Bayesian mixing model, and the deviation can be reduced by various means. Some research works estimated the degree of denitrification according to the Rayleigh equation $\delta_s = \delta_{s,0} - \epsilon_{s/p} \ln f$, or corrected the model by assuming the degree of denitrification (10%, 20%, 30%) (Yue et al., 2015). Some studies assumed the lowest $\delta^{18}\text{O}$ values of nitrate represent the original signature values (Jiang et al., 2021) or applied the constant enrichment factors directly to minimize

traceability error as much as possible (Kang and Xu, 2016; Xia et al., 2017). In addition, the means and standard deviations of nitrate isotopic characteristics in most studies were obtained from the literature (Zhang et al., 2018). Considering the different geochemical conditions and special regional activities, it is recommended to obtain the characteristic isotopic values ($\delta^{15}\text{N}$, $\delta^{18}\text{O}$) of nitrate produced in nitrification or derived from major pollutants by in-situ culture and measurement to improve the accuracy of source apportionment (Yu et al., 2020).

4.3 Models framed with enzyme-specific isotope effect

The implementation of isotopic fractionation in biogeochemical models will provide an additional evaluation parameter to improve the understanding of nitrate source and transformation processes (Denk et al., 2017). The successful integration of nitrogen isotopic composition into models requires a comprehensive understanding of the microscopic mechanism and basic data of enzyme-specific isotopic effect, and the quantification of uncertainties from different levels and incorporating them into the isotope model, so that the micro-scale mechanism and basic data can finally be applied in field practice.

The enzymatic reaction is the fundamental factor for isotope fractionations. It has been proved that there is a correlation between the abundance of enzyme functional genes and the enrichment of heavy isotopes ratio in natural ecosystems (Lennon and Houlton, 2017). Furthermore, although $^{18}\epsilon$ and $^{15}\epsilon$ significantly decreased from the enzyme scale to the ecosystem scale in nitrate transformation, the change degree of nitrogen and oxygen isotopes was consistent with the stable ratio of $^{18}\epsilon$ and $^{15}\epsilon$, and there were significant differences among different enzymes (e.g., Nar, Nap and eukNR), providing a cross-scale constant factor for nitrate identification. Considering the key role of enzymes in isotope fractionation and the consistency of enzyme-specific isotope effects across multiple scales, it is recommended to build isotope models framed with enzymes and their functional genes to interpret the field-observed results. However, the current models are mainly based on the microbial process other than on enzyme activity (Bai and Houlton, 2009; Denk et al., 2017; Somes et al., 2010). Moreover, it is difficult to guarantee the closed system under natural conditions, the inputs and outputs of the system exist simultaneously, in which most microbial processes occur in the mixed condition of closed systems, open systems, and infinite systems with no clear boundary. Finally, there are two main sources of uncertainties in isotope fractionation of nitrate: 1) biotic factors, including microbial assimilation, intermediates generation, and the influence of unknown biological processes. 2) abiotic factors, such as water exchange, volatilization of ammonia, volatilization and self-decomposition of nitrite, diffusion limitation, and unscientific sampling. Honestly, these deviations are hard to be avoided. Overall, it is difficult to effectively incorporate isotope fractionation into the models under the current research levels.

5. Conclusions and future perspectives

In conclusion, this paper revealed the mechanism and evidence of isotope fractionation from the perspective of ecology (enzyme, organism, population, community and ecosystem). The

exploration of the isotope fractionation mechanism will deepen the understanding of nitrate transformation, and the implement of isotope fractionation will further improve the accuracy in nitrate source identification. The major conclusions and perspectives in future research are as follows,

- 1) The enzymatic reaction is the fundamental factor for isotope fractionations. The enzyme-specific isotope effects ($^{15}\epsilon$ and $^{18}\epsilon$) during nitrification, denitrification and anammox are significant at small scales, which have been well summarized in this review (Table 1). The different affinity, electrical properties of the substrate channel and redox potential of the active sites for different enzymes may be key reason for the enzyme-specific isotope effects.
- 2) The proportionality of N and O isotope fractionation ($\delta^{18}\text{O}/\delta^{15}\text{N}$, $^{18}\epsilon/^{15}\epsilon$) associated with nitrate reduction in aquatic ecosystems generally follows enzyme-specific proportionalities (i.e., Nar, 0.95; Nap, 0.57; eukNR, 0.98) from small-scale investigations to large-scale ecosystems, providing enzyme-specific constant factors for the identification of nitrate transformation.
- 3) The significant isotope effects at small scales inevitably decrease in large-scale ecosystems due to the overlapping of microbial processes and the accumulation of uncertainties. To improve the accuracy of source apportionment, it is recommended to obtain the characteristic isotopic values ($\delta^{15}\text{N}$, $\delta^{18}\text{O}$) of nitrate produced in nitrification or derived from major pollutants by in-situ culture and measurement
- 4) Considering the enzyme-specific isotope effect, the isotope models based on enzymes is perhaps more promising than that based on microbial process. Future research needs to enhance the understanding of microscopic mechanisms, e.g., the theoretical calculation and the culture of microbial processes such as DNRA and Comammox under controlled laboratory conditions.

Acknowledgments

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References

- Anderson, L.J., Richardson, D.J., Butt, J.N., 2001. Catalytic Protein Film Voltammetry from a Respiratory Nitrate Reductase Provides Evidence for Complex Electrochemical Modulation of Enzyme Activity. *Biochemistry* 40, 11294–11307. <https://doi.org/10.1021/bi002706b>
- Arnoux, P., Sabaty, M., Alric, J., Frangioni, B., Guigliarelli, B., Adriano, J.-M., Pignol, D., 2003. Structural and redox plasticity in the heterodimeric periplasmic nitrate reductase. *Nat Struct Mol Biol* 10, 928–934. <https://doi.org/10.1038/nsb994>

- Asamoto, C.K., Rempfert, K.R., Luu, V.H., Younkin, A.D., Kopf, S.H., 2021. Enzyme-Specific Coupling of Oxygen and Nitrogen Isotope Fractionation of the Nap and Nar Nitrate Reductases. *Environ. Sci. Technol.* 55, 5537–5546. <https://doi.org/10.1021/acs.est.0c07816>
- Bai, E., Houlton, B.Z., 2009. Coupled isotopic and process-based modeling of gaseous nitrogen losses from tropical rain forests. *Global Biogeochemical Cycles* 23. <https://doi.org/10.1029/2008GB003361>
- Bigeleisen, J., Mayer, M.G., 1947. Calculation of Equilibrium Constants for Isotopic Exchange Reactions. *J. Chem. Phys.* 15, 261–267. <https://doi.org/10.1063/1.1746492>
- Boshers, D.S., Granger, J., Tobias, C.R., Böhlke, J.K., Smith, R.L., 2019. Constraining the Oxygen Isotopic Composition of Nitrate Produced by Nitrification. *Environ. Sci. Technol.* 53, 1206–1216. <https://doi.org/10.1021/acs.est.8b03386>
- Brunner, B., Contreras, S., Lehmann, M.F., Matantseva, O., Rollog, M., Kalvelage, T., Klockgether, G., Lavik, G., Jetten, M.S.M., Kartal, B., Kuypers, M.M.M., 2013. Nitrogen isotope effects induced by anammox bacteria. *Proceedings of the National Academy of Sciences* 110, 18994–18999. <https://doi.org/10.1073/pnas.1310488110>
- Buchwald, C., Casciotti, K.L., 2013. Isotopic ratios of nitrite as tracers of the sources and age of oceanic nitrite. *Nature Geosci* 6, 308–313. <https://doi.org/10.1038/ngeo1745>
- Buchwald, C., Casciotti, K.L., 2010. Oxygen isotopic fractionation and exchange during bacterial nitrite oxidation. *Limnology and Oceanography* 55, 1064–1074.
- Buchwald, C., Santoro, A.E., McIlvin, M.R., Casciotti, K.L., 2012. Oxygen isotopic composition of nitrate and nitrite produced by nitrifying cocultures and natural marine assemblages. *Limnology and Oceanography* 57, 1361–1375. <https://doi.org/10.4319/lo.2012.57.5.1361>
- Casciotti, K.L., 2009. Inverse kinetic isotope fractionation during bacterial nitrite oxidation. *Geochimica et Cosmochimica Acta* 73, 2061–2076. <https://doi.org/10.1016/j.gca.2008.12.022>
- Casciotti, K.L., McIlvin, M., Buchwald, C., 2010. Oxygen isotopic exchange and fractionation during bacterial ammonia oxidation. *Limnology and Oceanography* 55, 753–762. <https://doi.org/10.4319/lo.2010.55.2.0753>
- Casciotti, K.L., Sigman, D.M., Ward, B.B., 2003. Linking Diversity and Stable Isotope Fractionation in Ammonia-Oxidizing Bacteria. *Geomicrobiology Journal* 20, 335–353. <https://doi.org/10.1080/0149045030303895>
- Chen, F., Lao, Q., Zhang, S., Bian, P., Jin, G., Zhu, Q., Chen, C., 2020. Nitrate sources and biogeochemical processes identified using nitrogen and oxygen isotopes on the eastern coast of Hainan Island. *Continental Shelf Research* 207, 104209. <https://doi.org/10.1016/j.csr.2020.104209>
- Coelho, C., Romão, M.J., 2015. Structural and mechanistic insights on nitrate reductases. *Protein Sci* 24, 1901–1911. <https://doi.org/10.1002/pro.2801>

- 639 Coyne, M.S., Arunakumari, A., Averill, B.A., Tiedje, J.M., 1989. Immunological identification
640 and distribution of dissimilatory heme cd1 and nonheme copper nitrite reductases in
641 denitrifying bacteria. *Appl Environ Microbiol* 55, 2924–2931.
642 <https://doi.org/10.1128/aem.55.11.2924-2931.1989>
- 643 Delwiche, C.C., Steyn, P.L., 1970. Nitrogen isotope fractionation in soils and microbial reactions.
644 *Environ. Sci. Technol.* 4, 929–935. <https://doi.org/10.1021/es60046a004>
- 645 Denk, T.R.A., Mohn, J., Decock, C., Lewicka-Szczebak, D., Harris, E., Butterbach-Bahl, K., Kiese,
646 R., Wolf, B., 2017. The nitrogen cycle: A review of isotope effects and isotope modeling
647 approaches. *Soil Biology and Biochemistry* 105, 121–137.
648 <https://doi.org/10.1016/j.soilbio.2016.11.015>
- 649 Ellington, M.J.K., Bhakoo, K.K., Sawers, G., Richardson, D.J., Ferguson, S.J., 2002. Hierarchy of
650 carbon source selection in *Paracoccus pantotrophus*: strict correlation between reduction
651 state of the carbon substrate and aerobic expression of the nap operon. *J Bacteriol* 184, 4767–
652 4774. <https://doi.org/10.1128/JB.184.17.4767-4774.2002>
- 653 Fang, Y., Koba, K., Makabe, A., Zhu, F., Fan, S., Liu, X., Yoh, M., 2012. Low $\delta^{18}\text{O}$ Values of Nitrate
654 Produced from Nitrification in Temperate Forest Soils. *Environ. Sci. Technol.* 46, 8723–8730.
655 <https://doi.org/10.1021/es300510r>
- 656 Fenech, C., Rock, L., Nolan, K., Tobin, J., Morrissey, A., 2012. The potential for a suite of isotope
657 and chemical markers to differentiate sources of nitrate contamination: A review. *Water*
658 *Research* 46, 2023–2041. <https://doi.org/10.1016/j.watres.2012.01.044>
- 659 Frey, C., Hietanen, S., Jürgens, K., Labrenz, M., Voss, M., 2014. N and O Isotope Fractionation
660 in Nitrate during Chemolithoautotrophic Denitrification by *Sulfurimonas gotlandica*. *Environ.*
661 *Sci. Technol.* 48, 13229–13237. <https://doi.org/10.1021/es503456g>
- 662 Frisch, M.J., Trucks, G.W., Schlegel, H.B., Scuseria, G.E., Robb, M.A., Cheeseman, J.R., Scalmani,
663 G., Barone, V., Petersson, G.A., Nakatsuji, H., Li, X., Caricato, M., Marenich, A.V., Bloino, J.,
664 Janesko, B.G., Gomperts, R., Mennucci, B., Hratchian, H.P., Ortiz, J.V., Izmaylov, A.F.,
665 Sonnenberg, J.L., Williams-Young, D., Ding, F., Lipparini, F., Egidi, F., Goings, J., Peng, B., Petrone,
666 A., Henderson, T., Ranasinghe, D., Zakrzewski, V.G., Gao, J., Rega, N., Zheng, G., Liang, W., Hada,
667 M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao,
668 O., Nakai, H., Vreven, T., Throssell, K., Montgomery, J.A., Jr., Peralta, J.E., Ogliaro, F., Bearpark,
669 M.J., Heyd, J.J., Brothers, E.N., Kudin, K.N., Staroverov, V.N., Keith, T.A., Kobayashi, R., Normand,
670 J., Raghavachari, K., Rendell, A.P., Burant, J.C., Iyengar, S.S., Tomasi, J., Cossi, M., Millam, J.M.,
671 Klene, M., Adamo, C., Cammi, R., Ochterski, J.W., Martin, R.L., Morokuma, K., Farkas, O.,
672 Foresman, J.B., Fox, D.J., 2016. Gaussian~16 Revision C.01.
- 673 Fry, B., 2006. *Stable isotope ecology*. Springer, New York, NY.
- 674 Fülöp, V., Moir, J.W.B., Ferguson, S.J., Hajdu, J., 1995. The anatomy of a bifunctional enzyme:
675 Structural basis for reduction of oxygen to water and synthesis of nitric oxide by cytochrome
676 cd1. *Cell* 81, 369–377. [https://doi.org/10.1016/0092-8674\(95\)90390-9](https://doi.org/10.1016/0092-8674(95)90390-9)

- 677 Gates, A., Richardson, D., Butt, J., 2008. Voltammetric characterization of the aerobic energy-
 678 dissipating nitrate reductase of *Paracoccus pantotrophus*: exploring the activity of a redox-
 679 balancing enzyme as a function of electrochemical potential. *The Biochemical journal*.
 680 <https://doi.org/10.1042/BJ20071088>
- 681 Granger, J., Sigman, D.M., Lehmann, M.F., Tortell, P.D., 2008. Nitrogen and oxygen isotope
 682 fractionation during dissimilatory nitrate reduction by denitrifying bacteria. *Limnology and*
 683 *Oceanography* 53, 2533–2545. <https://doi.org/10.4319/lo.2008.53.6.2533>
- 684 Granger, J., Sigman, D.M., Needoba, J.A., Harrison, P.J., 2004. Coupled nitrogen and oxygen
 685 isotope fractionation of nitrate during assimilation by cultures of marine phytoplankton.
 686 *Limnology and Oceanography* 49, 1763–1773. <https://doi.org/10.4319/lo.2004.49.5.1763>
- 687 Granger, J., Sigman, D.M., Prokopenko, M.G., Lehmann, M.F., Tortell, P.D., 2006. A method for
 688 nitrite removal in nitrate N and O isotope analyses. *Limnology and Oceanography: Methods* 4,
 689 205–212. <https://doi.org/10.4319/lom.2006.4.205>
- 690 Granger, J., Wankel, S.D., 2016. Isotopic overprinting of nitrification on denitrification as a
 691 ubiquitous and unifying feature of environmental nitrogen cycling. *Proc Natl Acad Sci U S A*
 692 113, E6391–E6400. <https://doi.org/10.1073/pnas.1601383113>
- 693 He, Y., Zhang, Y., Zhang, S., Liu, Y., 2021. Predicting nitrogen and oxygen kinetic isotope effects
 694 of nitrate reduction by periplasmic dissimilatory nitrate reductase. *Geochimica et*
 695 *Cosmochimica Acta* 293, 224–239. <https://doi.org/10.1016/j.gca.2020.10.027>
- 696 Heil, J., Vereecken, H., Brüggemann, N., 2016. A review of chemical reactions of nitrification
 697 intermediates and their role in nitrogen cycling and nitrogen trace gas formation in soil.
 698 *European Journal of Soil Science* 67, 23–39. <https://doi.org/10.1111/ejss.12306>
- 699 Hu, Y., Jin, Z., Hu, Q., Hu, J., Ni, C., Li, F., 2020. Using stable isotopes to identify nitrogen
 700 transformations and estimate denitrification in a semi-constructed wetland. *Science of The*
 701 *Total Environment* 720, 137628. <https://doi.org/10.1016/j.scitotenv.2020.137628>
- 702 Jiang, H., Zhang, Q., Liu, W., Zhang, J., Zhao, T., Xu, Z., 2021. Climatic and anthropogenic driving
 703 forces of the nitrogen cycling in a subtropical river basin. *Environmental Research* 194, 110721.
 704 <https://doi.org/10.1016/j.envres.2021.110721>
- 705 Jormakka, M., Richardson, D., Byrne, B., Iwata, S., 2004. Architecture of NarGH reveals a
 706 structural classification of Mo-bisMGD enzymes. *Structure* 12, 95–104.
 707 <https://doi.org/10.1016/j.str.2003.11.020>
- 708 Kang, P., Xu, S., 2016. The impact of mariculture on nutrient dynamics and identification of the
 709 nitrate sources in coastal waters. *Environ Sci Pollut Res* 23, 1300–1311.
 710 <https://doi.org/10.1007/s11356-015-5363-0>
- 711 Kang, X., Niu, Y., Yu, H., Gou, P., Hou, Q., Lu, X., Wu, Y., 2022. Effect of rainfall-runoff process on
 712 sources and transformations of nitrate using a combined approach of dual isotopes,

- hydrochemical and Bayesian model in the Dagang River basin. *Science of The Total Environment* 837, 155674. <https://doi.org/10.1016/j.scitotenv.2022.155674>
- Karsh, K.L., Granger, J., Kritee, K., Sigman, D.M., 2012. Eukaryotic Assimilatory Nitrate Reductase Fractionates N and O Isotopes with a Ratio near Unity. *Environ. Sci. Technol.* 46, 5727–5735. <https://doi.org/10.1021/es204593q>
- Kaushal, S.S., Groffman, P.M., Band, L.E., Elliott, E.M., Shields, C.A., Kendall, C., 2011. Tracking Nonpoint Source Nitrogen Pollution in Human-Impacted Watersheds. *Environ. Sci. Technol.* 45, 8225–8232. <https://doi.org/10.1021/es200779e>
- Kendall, C., McDonnell, J.J., 2012. *Isotope tracers in catchment hydrology*. Elsevier.
- Knöller, K., Vogt, C., Haupt, M., Feisthauer, S., Richnow, H.-H., 2011. Experimental investigation of nitrogen and oxygen isotope fractionation in nitrate and nitrite during denitrification. *Biogeochemistry* 103, 371–384. <https://doi.org/10.1007/s10533-010-9483-9>
- Kobayashi, K., Makabe, A., Yano, M., Oshiki, M., Kindaichi, T., Casciotti, K.L., Okabe, S., 2019. Dual nitrogen and oxygen isotope fractionation during anaerobic ammonium oxidation by anammox bacteria. *The ISME Journal* 13, 2426–2436. <https://doi.org/10.1038/s41396-019-0440-x>
- Kotajima, S., Koba, K., Ikeda, D., Terada, A., Isaka, K., Nishina, K., Kimura, Y., Makabe, A., Yano, M., Fujitani, H., Ushiki, N., Tsuneda, S., Yoh, M., 2020. Nitrogen and Oxygen Isotope Signatures of Nitrogen Compounds during Anammox in the Laboratory and a Wastewater Treatment Plant. *Microbes and Environments* 35. <https://doi.org/10.1264/jsme2.ME20031>
- Kuypers, M.M.M., Marchant, H.K., Kartal, B., 2018. The microbial nitrogen-cycling network. *Nat Rev Microbiol* 16, 263–276. <https://doi.org/10.1038/nrmicro.2018.9>
- Lennon, E.F.E., Houlton, B.Z., 2017. Coupled molecular and isotopic evidence for denitrifier controls over terrestrial nitrogen availability. *The ISME Journal* 11, 727–740. <https://doi.org/10.1038/ismej.2016.147>
- Li, C., Li, S.-L., Yue, F.-J., Liu, J., Zhong, J., Yan, Z.-F., Zhang, R.-C., Wang, Z.-J., Xu, S., 2019. Identification of sources and transformations of nitrate in the Xijiang River using nitrate isotopes and Bayesian model. *Science of The Total Environment* 646, 801–810. <https://doi.org/10.1016/j.scitotenv.2018.07.345>
- Li, J., Zhu, D., Zhang, S., Yang, G., Zhao, Y., Zhou, C., Lin, Y., Zou, S., 2022. Application of the hydrochemistry, stable isotopes and MixSIAR model to identify nitrate sources and transformations in surface water and groundwater of an intensive agricultural karst wetland in Guilin, China. *Ecotoxicology and Environmental Safety* 231, 113205. <https://doi.org/10.1016/j.ecoenv.2022.113205>
- Liu, C.-Q., Li, S.-L., Lang, Y.-C., Xiao, H.-Y., 2006. Using $\delta^{15}\text{N}$ - and $\delta^{18}\text{O}$ -Values To Identify Nitrate Sources in Karst Ground Water, Guiyang, Southwest China. *Environ. Sci. Technol.* 40, 6928–6933. <https://doi.org/10.1021/es0610129>

- 750 Lorette, G., Sebilo, M., Buquet, D., Lastennet, R., Denis, A., Peyraube, N., Charriere, V., Studer,
751 J.-C., 2022. Tracing sources and fate of nitrate in multilayered karstic hydrogeological
752 catchments using natural stable isotopic composition ($\delta^{15}\text{N-NO}_3^-$ and $\delta^{18}\text{O-NO}_3^-$).
753 Application to the Toulon karst system (Dordogne, France). *Journal of Hydrology* 610, 127972.
754 <https://doi.org/10.1016/j.jhydrol.2022.127972>
- 755 Marangon, J., Paes de Sousa, P.M., Moura, I., Brondino, C.D., Moura, J.J.G., González, P.J., 2012.
756 Substrate-dependent modulation of the enzymatic catalytic activity: Reduction of nitrate,
757 chlorate and perchlorate by respiratory nitrate reductase from *Marinobacter*
758 *hydrocarbonoclasticus* 617. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1817, 1072–
759 1082. <https://doi.org/10.1016/j.bbabi.2012.04.011>
- 760 Mariotti, A., Germon, J.C., Hubert, P., Kaiser, P., Letolle, R., Tardieux, A., Tardieux, P., 1981.
761 Experimental determination of nitrogen kinetic isotope fractionation: Some principles;
762 illustration for the denitrification and nitrification processes. *Plant Soil* 62, 413–430.
763 <https://doi.org/10.1007/BF02374138>
- 764 Martin, T.S., Casciotti, K.L., 2016. Nitrogen and oxygen isotopic fractionation during microbial
765 nitrite reduction. *Limnology and Oceanography* 61, 1134–1143.
766 <https://doi.org/10.1002/lno.10278>
- 767 Michener, R.H., Lajtha, K. (Eds.), 2007. Stable isotopes in ecology and environmental science,
768 2nd ed. ed, Ecological methods and concepts series. Blackwell Pub, Malden, MA.
- 769 Moir, J.W., Wood, N.J., 2001. Nitrate and nitrite transport in bacteria. *Cell Mol Life Sci* 58, 215–
770 224. <https://doi.org/10.1007/PL00000849>
- 771 Mooshammer, M., Alves, R.J.E., Bayer, B., Melcher, M., Stieglmeier, M., Jochum, L., Rittmann,
772 S.K.-M.R., Watzka, M., Schleper, C., Herndl, G.J., Wanek, W., 2020. Nitrogen Isotope
773 Fractionation During Archaeal Ammonia Oxidation: Coupled Estimates From Measurements
774 of Residual Ammonium and Accumulated Nitrite. *Front. Microbiol.* 11.
775 <https://doi.org/10.3389/fmicb.2020.01710>
- 776 Murphy, M.E.P., Turley, S., Adman, E.T., 1997. Structure of Nitrite Bound to Copper-containing
777 Nitrite Reductase from *Alcaligenes faecalis*: MECHANISTIC IMPLICATIONS *. *Journal of*
778 *Biological Chemistry* 272, 28455–28460. <https://doi.org/10.1074/jbc.272.45.28455>
- 779 Najmudin, S., González, P.J., Trincão, J., Coelho, C., Mukhopadhyay, A., Cerqueira, N.M.F.S.A.,
780 Romão, C.C., Moura, I., Moura, J.J.G., Brondino, C.D., Romão, M.J., 2008. Periplasmic nitrate
781 reductase revisited: a sulfur atom completes the sixth coordination of the catalytic
782 molybdenum. *J Biol Inorg Chem* 13, 737–753. <https://doi.org/10.1007/s00775-008-0359-6>
- 783 Pinho, D., Besson, S., Silva, P.J., de Castro, B., Moura, I., 2005. Isolation and spectroscopic
784 characterization of the membrane-bound nitrate reductase from *Pseudomonas chlororaphis*
785 DSM 50135. *Biochim Biophys Acta* 1723, 151–162.
786 <https://doi.org/10.1016/j.bbagen.2005.02.008>

- 787 Ryu, Kang, T.-W., Kim, K., Nam, T.-H., Han, Y., Kim, J., Kim, M.-S., Lim, H., Seo, K.-A., Lee, K., Yoon,
788 S.-H., Hwang, S.H., Na, E.H., Lee, J.H., 2021a. Tracking nitrate sources in agricultural-urban
789 watershed using dual stable isotope and Bayesian mixing model approach: Considering N
790 transformation by Lagrangian sampling. *Journal of Environmental Management* 300, 113693.
791 <https://doi.org/10.1016/j.jenvman.2021.113693>
- 792 Ryu, Kim, S.-J., Baek, U., Kim, D.-W., Lee, H.-J., Chung, E.G., Kim, M.-S., Kim, K., Lee, J.K., 2021b.
793 Identifying nitrogen sources in intensive livestock farming watershed with swine excreta
794 treatment facility using dual ammonium ($\delta^{15}\text{NNH}_4$) and nitrate ($\delta^{15}\text{NNO}_3$) nitrogen isotope
795 ratios axes. *Science of The Total Environment* 779, 146480.
796 <https://doi.org/10.1016/j.scitotenv.2021.146480>
- 797 Santoro, A.E., Casciotti, K.L., 2011. Enrichment and characterization of ammonia-oxidizing
798 archaea from the open ocean: phylogeny, physiology and stable isotope fractionation. *ISME J*
799 5, 1796–1808. <https://doi.org/10.1038/ismej.2011.58>
- 800 Sears, H.J., Sawers, G., Berks, B.C., Ferguson, S.J., Richardson, D.J., 2000. Control of periplasmic
801 nitrate reductase gene expression (napEDABC) from *Paracoccus pantotrophus* in response to
802 oxygen and carbon substrates. *Microbiology (Reading)* 146 (Pt 11), 2977–2985.
803 <https://doi.org/10.1099/00221287-146-11-2977>
- 804 Shi, Y., Li, C., Jin, Z., Zhang, Y., Xiao, J., Li, F., 2021. Combining dual isotopes and a Bayesian
805 isotope mixing model to quantify the nitrate sources of precipitation in Ningbo, East China.
806 *Science of The Total Environment* 778, 146297.
807 <https://doi.org/10.1016/j.scitotenv.2021.146297>
- 808 Somes, C.J., Schmittner, A., Galbraith, E.D., Lehmann, M.F., Altabet, M.A., Montoya, J.P.,
809 Letelier, R.M., Mix, A.C., Bourbonnais, A., Eby, M., 2010. Simulating the global distribution of
810 nitrogen isotopes in the ocean. *Global Biogeochemical Cycles* 24.
811 <https://doi.org/10.1029/2009GB003767>
- 812 Sparacino-Watkins, C., Stolz, J.F., Basu, P., 2014. Nitrate and periplasmic nitrate reductases.
813 *Chem Soc Rev* 43, 676–706. <https://doi.org/10.1039/c3cs60249d>
- 814 Spiro, S., Guest, J.R., 1990. FNR and its role in oxygen-regulated gene expression in *Escherichia*
815 *coli*. *FEMS Microbiol Rev* 6, 399–428. <https://doi.org/10.1111/j.1574-6968.1990.tb04109.x>
- 816 Stein, L.Y., Klotz, M.G., 2016. The nitrogen cycle. *Current Biology* 26, R94–R98.
817 <https://doi.org/10.1016/j.cub.2015.12.021>
- 818 Stern, M., Wolfsberg, M., 1966. Simplified Procedure for the Theoretical Calculation of Isotope
819 Effects Involving Large Molecules. <https://doi.org/10.1063/1.1727463>
- 820 Strous, M., Pelletier, E., Mangenot, S., Rattei, T., Lehner, A., Taylor, M.W., Horn, M., Daims, H.,
821 Bartol-Mavel, D., Wincker, P., Barbe, V., Fonknechten, N., Vallenet, D., Segurens, B.,
822 Schenowitz-Truong, C., Médigue, C., Collingro, A., Snel, B., Dutilh, B.E., Op den Camp, H.J.M.,
823 van der Drift, C., Cirpus, I., van de Pas-Schoonen, K.T., Harhangi, H.R., van Niftrik, L., Schmid,

- 824 M., Keltjens, J., van de Vossenberg, J., Kartal, B., Meier, H., Frishman, D., Huynen, M.A., Mewes,
825 H.-W., Weissenbach, J., Jetten, M.S.M., Wagner, M., Le Paslier, D., 2006. Deciphering the
826 evolution and metabolism of an anammox bacterium from a community genome. *Nature* 440,
827 790–794. <https://doi.org/10.1038/nature04647>
- 828 Torres-Martínez, J.A., Mora, A., Knappett, P.S.K., Ornelas-Soto, N., Mahlknecht, J., 2020.
829 Tracking nitrate and sulfate sources in groundwater of an urbanized valley using a multi-tracer
830 approach combined with a Bayesian isotope mixing model. *Water Research* 182, 115962.
831 <https://doi.org/10.1016/j.watres.2020.115962>
- 832 Treibergs, L.A., Granger, J., 2017. Enzyme level N and O isotope effects of assimilatory and
833 dissimilatory nitrate reduction. *Limnology and Oceanography* 62, 272–288.
834 <https://doi.org/10.1002/lno.10393>
- 835 Urey, H.C., 1947. The thermodynamic properties of isotopic substances. *J. Chem. Soc.* 562–581.
836 <https://doi.org/10.1039/JR9470000562>
- 837 Wells, N.S., Hakoun, V., Brouyère, S., Knöller, K., 2016. Multi-species measurements of nitrogen
838 isotopic composition reveal the spatial constraints and biological drivers of ammonium
839 attenuation across a highly contaminated groundwater system. *Water Research* 98, 363–375.
840 <https://doi.org/10.1016/j.watres.2016.04.025>
- 841 Wolfsberg, M., Stern, M., 1964. Validity of some approximation procedures used in the
842 theoretical calculation of isotope effects. <https://doi.org/10.1351/PAC196408030225>
- 843 Wong, W.W., Grace, M.R., Cartwright, I., Cook, P.L.M., 2014. Sources and fate of nitrate in a
844 groundwater-fed estuary elucidated using stable isotope ratios of nitrogen and oxygen.
845 *Limnology and Oceanography* 59, 1493–1509. <https://doi.org/10.4319/lo.2014.59.5.1493>
- 846 Wood, N.J., Alizadeh, T., Bennett, S., Pearce, J., Ferguson, S.J., Richardson, D.J., Moir, J.W., 2001.
847 Maximal expression of membrane-bound nitrate reductase in *Paracoccus* is induced by nitrate
848 via a third FNR-like regulator named NarR. *J Bacteriol* 183, 3606–3613.
849 <https://doi.org/10.1128/JB.183.12.3606-3613.2001>
- 850 Wu, Z., Yu, Z., Song, X., Wang, W., Zhou, P., Cao, X., Yuan, Y., 2019. Key nitrogen biogeochemical
851 processes in the South Yellow Sea revealed by dual stable isotopes of nitrate. *Estuarine, Coastal*
852 *and Shelf Science* 225, 106222. <https://doi.org/10.1016/j.ecss.2019.05.004>
- 853 Wunderlich, A., Meckenstock, R., Einsiedl, F., 2012. Effect of Different Carbon Substrates on
854 Nitrate Stable Isotope Fractionation During Microbial Denitrification. *Environ. Sci. Technol.* 46,
855 4861–4868. <https://doi.org/10.1021/es204075b>
- 856 Xia, Y., Li, Y., Zhang, X., Yan, X., 2017. Nitrate source apportionment using a combined dual
857 isotope, chemical and bacterial property, and Bayesian model approach in river systems.
858 *Journal of Geophysical Research: Biogeosciences* 122, 2–14.
859 <https://doi.org/10.1002/2016JG003447>

- 860 Xuan, Y., Liu, G., Zhang, Y., Cao, Y., 2022. Factor affecting nitrate in a mixed land-use watershed
861 of southern China based on dual nitrate isotopes, sources or transformations? *Journal of*
862 *Hydrology* 604, 127220. <https://doi.org/10.1016/j.jhydrol.2021.127220>
- 863 Xuan, Y., Tang, C., Cao, Y., 2020. Mechanisms of nitrate accumulation in highly urbanized rivers:
864 Evidence from multi-isotopes in the Pearl River Delta, China. *Journal of Hydrology* 587, 124924.
865 <https://doi.org/10.1016/j.jhydrol.2020.124924>
- 866 Xue, D., Botte, J., De Baets, B., Accoe, F., Nestler, A., Taylor, P., Van Cleemput, O., Berglund, M.,
867 Boeckx, P., 2009. Present limitations and future prospects of stable isotope methods for nitrate
868 source identification in surface- and groundwater. *Water Research* 43, 1159–1170.
869 <https://doi.org/10.1016/j.watres.2008.12.048>
- 870 Yu, L., Zheng, T., Zheng, X., Hao, Y., Yuan, R., 2020. Nitrate source apportionment in
871 groundwater using Bayesian isotope mixing model based on nitrogen isotope fractionation.
872 *Science of The Total Environment* 718, 137242.
873 <https://doi.org/10.1016/j.scitotenv.2020.137242>
- 874 Yue, F.-J., Li, S.-L., Hu, J., 2015. The Contribution of Nitrate Sources in Liao Rivers, China, Based
875 on Isotopic Fractionation and Bayesian Mixing Model. *Procedia Earth and Planetary Science*
876 13, 16–20. <https://doi.org/10.1016/j.proeps.2015.07.004>
- 877 Zaryab, A., Nassery, H.R., Knoeller, K., Alijani, F., Minet, E., 2022. Determining nitrate pollution
878 sources in the Kabul Plain aquifer (Afghanistan) using stable isotopes and Bayesian stable
879 isotope mixing model. *Science of The Total Environment* 823, 153749.
880 <https://doi.org/10.1016/j.scitotenv.2022.153749>
- 881 Zhang, J., Cao, M., Jin, M., Huang, X., Zhang, Z., Kang, F., 2022. Identifying the source and
882 transformation of riverine nitrates in a karst watershed, North China: Comprehensive use of
883 major ions, multiple isotopes and a Bayesian model. *Journal of Contaminant Hydrology* 246,
884 103957. <https://doi.org/10.1016/j.jconhyd.2022.103957>
- 885 Zhang, M., Zhi, Y., Shi, J., Wu, L., 2018. Apportionment and uncertainty analysis of nitrate
886 sources based on the dual isotope approach and a Bayesian isotope mixing model at the
887 watershed scale. *Science of The Total Environment* 639, 1175–1187.
888 <https://doi.org/10.1016/j.scitotenv.2018.05.239>
- 889 Zhang, X., Ward, B.B., Sigman, D.M., 2020. Global Nitrogen Cycle: Critical Enzymes, Organisms,
890 and Processes for Nitrogen Budgets and Dynamics. *Chem. Rev.* 120, 5308–5351.
891 <https://doi.org/10.1021/acs.chemrev.9b00613>
- 892

Highlights

Ignorance of isotope fractionations impedes the nitrate source identification;

Enzymatic reaction is the fundamental factor to drive isotope fractionations;

$^{18}\text{E}/^{15}\text{E}$ provides enzyme-specific constant factors for nitrate source identification;

Appropriate practices of isotopic effect improves the accuracy of identification.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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