



Biogeochemical and hydrological controls on the oxygen isotopic composition of nitrification-produced nitrate in an agricultural soil

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Abstract. The oxygen isotopic composition of nitrate (NO_3^-) produced by nitrification ($\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$) establishes the initial isotopic signature of NO_3^- in environmental systems and provides a baseline for tracing NO_3^- sources and subsequent transformations. However, how $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ and its relationship with water $\delta^{18}\text{O}$ ($\delta^{18}\text{O}_{\text{H}_2\text{O}}$) vary with temperature and substrate availability in agricultural soils remains poorly constrained. Moreover, soil evaporation can enrich $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in surface soils where nitrification is most active, yet how this enrichment influences $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ under contrasting hydrological conditions remains unexplored. To address these gaps, we integrated soil incubation experiments with a synthetic transport modeling framework based on StorAge Selection (SAS) functions to quantify how biogeochemical and hydrological factors regulate the variability and predictability of $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ in an agricultural soil. The incubation experiments showed that, across wide ranges of temperature (7–35 °C) and ammonium (NH_4^+) availability (0–98 mg N kg⁻¹ soil), $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ generally tracked soil $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ but varied by 3.4–5.3‰ at a given $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. Temperature and NH_4^+ availability influenced $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ primarily by regulating transient nitrite (NO_2^-) accumulation, which enhanced NO_2^- –water oxygen isotope exchange and thereby altered the expression of the associated equilibrium isotope effect. Synthetic transport modeling further revealed that hydrological processes can generate $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ variability comparable in magnitude to that produced by biogeochemical controls. Because $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ records the $\delta^{18}\text{O}$ of water in nitrification zones at the time of NO_3^- production, whereas drainage $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ is additionally shaped by the mixing of water transported through heterogeneous flow paths, using drainage $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ to predict $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ can produce systematic biases, particularly when seasonal nitrification dynamics interact with soil evaporation and water transport. Overall, our results show that $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ in soil is jointly controlled by biogeochemical processes that regulate the expression of kinetic and equilibrium isotope effects during nitrification and by hydrological processes that determine the sources, ages, and flow paths of water supplying nitrification. These findings underscore the need to move beyond stream-centric approaches toward reactive transport frameworks that explicitly accounts for dynamic biogeochemical controls across diverse flow paths and water ages when applying NO_3^- isotopes to environmental systems.

1 Introduction

Stable isotope ratios in nitrate (NO_3^-) provide valuable tracers of nitrogen (N) sources and transformation processes in terrestrial and aquatic systems (Kendall et al., 2007; Xu et al., 2016; Venkiteswaran et al., 2019; Matiatos et al., 2021). Among these processes, microbial nitrification – the oxidation of ammoniacal N to NO_3^- – is a dominant source of NO_3^- in many terrestrial and aquatic systems, particularly those influenced by anthropogenic N inputs. As a result, the N and oxygen (O) isotopic composition of nitrification-derived NO_3^- establishes the initial isotopic signature of NO_3^- and provides the baseline for NO_3^- source apportionment and interpretation of subsequent transformations such as denitrification.

During nitrification, O atoms from ambient water are incorporated into newly formed NO_3^- , making the O isotope ratio of environmental water ($\delta^{18}\text{O}_{\text{H}_2\text{O}}$) a direct control on the O isotopic composition of nitrification-derived NO_3^- ($\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$) (Casciotti et al., 2010; Buchwald and Casciotti, 2010). At the same time, $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ is widely used as a hydrological tracer to infer water sources,



flow paths, and transport processes that govern NO_3^- mobilization and export (Kirchner et al., 2010; Li et al., 2021). This dual role creates a direct isotopic linkage between NO_3^- production and hydrological transport, offering a framework to study coupled N cycling and water movement in complex environmental systems.

- 40 In field studies, $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ is commonly estimated using a stoichiometric framework that assumes a fixed 2:1 contribution of O atoms from water and atmospheric O_2 during nitrification (Kendall et al., 2007). However, this assumption can be violated because both abiotic and biologically mediated O exchange between nitrite (NO_2^-) – an intermediate in nitrification – and water may increase the contribution of water-derived O beyond this nominal ratio (Bonner, 1970; Andersson et al., 1982). Furthermore, $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ reflects both kinetic isotope effects during enzymatic reactions and equilibrium isotope effects associated with O
- 45 exchange between NO_2^- and H_2O (see Section 2.1) (Casciotti et al., 2010; Buchwald and Casciotti, 2010). Despite these complexities, incubation studies across bacterial and archaeal cultures, as well as seawater, freshwater, and soil systems, consistently show that $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ exhibits an approximately 1:1 relationship with $\delta^{18}\text{O}_{\text{H}_2\text{O}}$, suggesting compensating fractionation effects during nitrification (Casciotti et al., 2002; Buchwald and Casciotti, 2010; Snider et al., 2010; Buchwald et al., 2012; Boshers et al., 2019; Hu et al., 2024).
- 50 A key limitation of these previous studies is that they were conducted under relatively constrained environmental conditions, such as constant temperature or fixed initial substrate supply. In agricultural soils receiving fertilizer and manure inputs, however, both N availability and temperature vary substantially and can exert a substantial control on nitrification dynamics. Our previous laboratory incubation study demonstrated that increased substrate availability can elevate $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ under constant temperature conditions, likely through enhanced O isotope exchange during nitrification (Hu et al., 2024). Temperature may further regulate
- 55 $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ by altering both intrinsic isotope effects and reaction kinetics. This may occur directly through temperature-dependent shifts in isotopic partitioning associated with molecular vibrational energies (Urey, 1947; Bigeleisen and Wolfsberg, 1958), and indirectly through changes in reaction rates that alter the rate-limiting step of multi-step reactions, thereby influencing the extent to which intrinsic isotope effects are expressed (Canfield et al., 2006). To our knowledge, the combined effects of substrate availability and temperature on $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$, and on its relationship with environmental $\delta^{18}\text{O}_{\text{H}_2\text{O}}$, have not been quantified.
- 60 Because soil water supplies the O atoms incorporated into microbially produced NO_3^- , $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ is intrinsically linked not only to biogeochemical processes but also to soil hydrological dynamics. Near the soil surface, where nitrification is most active, evaporation can enrich heavy isotopes in soil water (Craig and Gordon, 1965), potentially increasing $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ independent of biogeochemical controls. At the same time, subsurface heterogeneity can generate distinct flow paths across pore-to-landscape scales, separating water used for evapotranspiration from water contributing to drainage and streamflow (Sprenger et al., 2016).
- 65 This phenomenon, often described as ecohydrological separation (Brooks et al., 2010; McDonnell, 2014), introduces uncertainty in watershed-scale applications where streamflow $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ is commonly used to estimate $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$. Such estimates implicitly assume complete subsurface mixing, an assumption that may not hold in heterogeneous systems.
- To better understand hydrological controls on NO_3^- export and isotope dynamics, our previous studies applied StorAge Selection (SAS) functions to estimate time-varying water transit times in intensively managed agricultural systems (Yu et al., 2023; Sha et al., 2025). SAS functions provide a parsimonious representation of subsurface transport processes (see Section 2.2) (Benettin et al., 2022), enabling analysis of how hydrological transport interacts with NO_3^- isotope dynamics. These studies revealed strong relationships between water age and the O isotopic composition of NO_3^- ($\delta^{18}\text{O}_{\text{NO}_3}$) in soil drainage and streamflow. Specifically, low $\delta^{18}\text{O}_{\text{NO}_3}$ values, indicative of nitrification, were strongly associated with young water during high-flow conditions, whereas elevated $\delta^{18}\text{O}_{\text{NO}_3}$ values reflected enhanced denitrification in older water (Yu et al., 2023; Sha et al., 2025). Building on these
- 75 findings, a water age-based NO_3^- transport model was developed to quantify source availability and denitrification through joint simulation of $\delta^{18}\text{O}_{\text{NO}_3}$ and water age. However, a major limitation of this framework is that $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ – the baseline isotopic



signature for evaluating denitrification enrichment – was treated as a fixed calibrated parameter due to limited mechanistic constraints.

This study aims to resolve the biogeochemical and hydrological controls on $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ in an agricultural soil to improve the application of coupled NO_3^- and water isotope tracers. Specifically, we address two questions:

(1) How do temperature and substrate availability regulate the variability of $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ and its relationship with environmental $\delta^{18}\text{O}_{\text{H}_2\text{O}}$?

(2) How does evaporative enrichment influence $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ under different water transport regimes in the soil source zone?

To address these questions, we conducted static soil incubation experiments across gradients of temperature and ammonium (NH_4^+)

availability to quantify controls on $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ and its relationship with $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. We then developed a biogeochemical model based on the Michaelis–Menten–Monod framework to simulate NO_3^- production and isotope dynamics, enabling mechanistic evaluation of temperature and kinetic effects. The simulated $\delta^{18}\text{O}_{\text{H}_2\text{O}}-\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ relationships were subsequently integrated into a synthetic transport framework based on SAS functions. The purpose of this integration was not to reproduce site-specific hydrological dynamics but to use synthetic water fluxes as a controlled platform for investigating how evaporative enrichment and flow path heterogeneity influence the variability and predictability of $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$.

The remainder of this manuscript is organized as follows. Because the study integrates O isotope systematics during nitrification with transport and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ simulations based on SAS functions, Section 2 summarizes the theoretical foundations of both frameworks. Section 3 describes the laboratory incubation experiments and development of the biogeochemical model for simulating $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$. Section 4 describes the synthetic transport simulations used to investigate the effects of evaporation and water transport on $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ and its prediction using $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. Section 5 presents the results, followed by Section 6, which discusses the key findings and their implications for using coupled NO_3^- and water isotopes to trace NO_3^- reactive transport.

2 Background Theory

2.1 Oxygen Isotope Systematics of Nitrification

Nitrification proceeds through two sequential steps, each involving distinct pathways of O incorporation and associated isotope effects (Fig. 1). By convention, O isotope ratios are reported using δ notation, where $\delta^{18}\text{O} = ([^{18}\text{O}/^{16}\text{O}]_{\text{sample}} / [^{18}\text{O}/^{16}\text{O}]_{\text{VSMOW}} - 1) \times 1000$, in units of per mille (‰). In the first step, ammonia (NH_3) is oxidized to NO_2^- by NH_3 oxidizing bacteria (AOB) and archaea (AOA), during which O atoms are incorporated from both O_2 and H_2O . These incorporations are associated with kinetic O isotope effects ($^{18}\epsilon_{\text{k}, \text{H}_2\text{O}, 1}$ and $^{18}\epsilon_{\text{k}, \text{O}_2}$, in ‰), which lower the $\delta^{18}\text{O}$ of incorporated O relative to the source O_2 and H_2O pools (Fig. 1). During the second step, NO_2^- is oxidized to NO_3^- by NO_2^- oxidizing bacteria (NOB), with the additional O atom derived from water (Fig. 1). This step is associated with further kinetic isotope effects related to O incorporation and NO_2^- consumption ($^{18}\epsilon_{\text{k}, \text{H}_2\text{O}, 2}$ and $^{18}\epsilon_{\text{k}, \text{NO}}$).

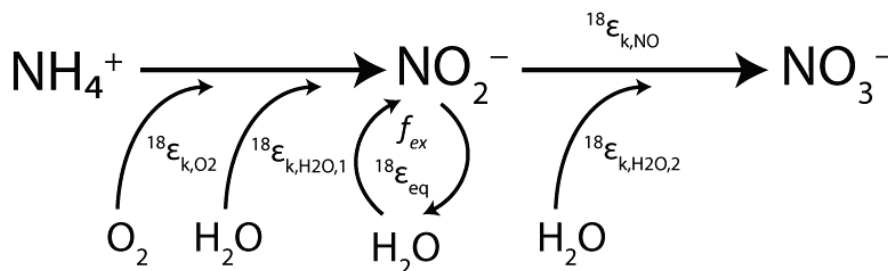


Figure 1. Oxygen isotope systematics during nitrification. Sources of O atoms (H₂O and O₂) for NH₄⁺ and NO₂⁻ oxidation and the associated isotope effects (¹⁸ε_{k,O2}, ¹⁸ε_{k,H2O,1}, ¹⁸ε_{k,H2O,2}, ¹⁸ε_{k,NO}) are indicated. The fraction of NO₂⁻ O atoms exchanged with water (*f_{ex}*) and the associated equilibrium isotope effect (¹⁸ε_{eq}) are also shown. Adapted from Boshers et al. (2019).

In addition to these kinetic isotope effects, O atoms in NO₂⁻ can exchange isotopically with water, a process governed by the equilibrium isotope effect (¹⁸ε_{eq}) (Fig. 1). At isotopic equilibrium, this exchange enriches NO₂⁻ in ¹⁸O relative to water, such that the δ¹⁸O of NO₂⁻ exceeds δ¹⁸O_{H2O} by approximately 14‰ at 17 °C (i.e., ¹⁸ε_{eq} ≈ 14‰) (Buchwald and Casciotti, 2013). Previous incubation studies have shown that both AOB and AOA can catalyze this O isotope exchange during NH₃ oxidation (Casciotti et al., 2010; Nishizawa et al., 2016), whereas O exchange during NO₂⁻ oxidation is generally considered negligible (Hollocher, 1984; DiSpirito and Hooper, 1986; Buchwald and Casciotti, 2010). In addition to this biologically mediated O isotope exchange, abiotic O isotope exchange between NO₂⁻ and water is thermodynamically favorable and can proceed rapidly under acidic conditions (Casciotti et al., 2007).

Based on this current understanding, an O isotope mass balance model has been developed to relate δ¹⁸O_{NO3,nit} to the δ¹⁸O of its O sources (O₂ and water) and the associated kinetic and equilibrium isotope effects (Buchwald et al., 2012):

$$\delta^{18}\text{O}_{\text{NO3,nit}} = \frac{2}{3} \left\{ \left[\frac{1}{2} (\delta^{18}\text{O}_{\text{O2}} - {}^{18}\epsilon_{\text{k,O2}}) + \frac{1}{2} (\delta^{18}\text{O}_{\text{H2O}} - {}^{18}\epsilon_{\text{k,H2O,1}}) \right] (1 - f_{\text{ex}}) + (\delta^{18}\text{O}_{\text{H2O}} + {}^{18}\epsilon_{\text{eq}}) f_{\text{ex}} \right\} + \frac{1}{3} (\delta^{18}\text{O}_{\text{H2O}} - {}^{18}\epsilon_{\text{k,H2O,2}}) \quad (1)$$

where δ¹⁸O_{O2} is the isotopic composition of O₂, and *f_{ex}* represents the fraction of NO₂⁻ that undergoes O isotope exchange with water before oxidation to NO₃⁻.

Previous studies have shown that the δ¹⁸O of O₂ in a wide range of surface soils, including highly productive rainforest soils (Angert et al., 2003), varies only slightly (±1‰) from atmospheric O₂ (23.5‰) (Angert et al., 2003; Spoelstra et al., 2007). Accordingly, assuming a relatively constant δ¹⁸O_{O2}, Eq. 1 is rearranged to express δ¹⁸O_{NO3,nit} as a linear function of δ¹⁸O_{H2O}:

$$\delta^{18}\text{O}_{\text{NO3,nit}} = \left(\frac{2}{3} + \frac{1}{3} f_{\text{ex}} \right) \delta^{18}\text{O}_{\text{H2O}} + \frac{1}{3} [(\delta^{18}\text{O}_{\text{O2}} - {}^{18}\epsilon_{\text{k,comp}})(1 - f_{\text{ex}}) - {}^{18}\epsilon_{\text{k,H2O,2}}] + \frac{2}{3} f_{\text{ex}} {}^{18}\epsilon_{\text{eq}} \quad (2)$$

where ¹⁸ε_{k,comp} is the composite kinetic isotope effect associated with O incorporation during NH₄⁺ oxidation (i.e., ¹⁸ε_{k,H2O,1} + ¹⁸ε_{k,O2}). The slope and intercept of this linear relationship are determined by *f_{ex}* and the isotope effects ¹⁸ε_{k,comp}, ¹⁸ε_{k,H2O,2}, and ¹⁸ε_{eq}. With prior information on ¹⁸ε_{eq}, these parameters can be inferred from regressions between δ¹⁸O_{NO3,nit} and δ¹⁸O_{H2O} in incubation experiments employing replicate treatments with different δ¹⁸O_{H2O} values.

Reported ranges of *f_{ex}*, ¹⁸ε_{k,comp}, and ¹⁸ε_{k,H2O,2} from these experiments across culture, seawater, freshwater, and soil systems are summarized in Table 1. In addition, ¹⁸ε_{k,NO} has been estimated from kinetic NO₂⁻ consumption experiments (Buchwald and Casciotti, 2010). Based on these ranges and experimentally determined ¹⁸ε_{eq} values, δ¹⁸O_{NO3,nit} is predicted to fall within ±3‰ of δ¹⁸O_{H2O} (Boshers et al., 2019). This relatively narrow range reflects compensating effects among (i) the elevated δ¹⁸O of atmospheric O₂, (ii) kinetic isotope effects that lower the δ¹⁸O of incorporated O, and (iii) O isotope exchange between NO₂⁻ and water, which both increases the proportion of water-derived O atom and enriches ¹⁸O in NO₂⁻.



140 It is important to note that Eq. 1 and 2 assume complete consumption of the intermediate NO_2^- pool, allowing $^{18}\epsilon_{\text{k,NO}}$ to be omitted from the formulation of $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$. Previous pure culture studies have shown that NO_2^- oxidation can exhibit an inverse O isotope effect, whereby heavier NO_2^- isotopologues are preferentially consumed (Buchwald and Casciotti, 2010). Consequently, when NO_2^- accumulates during nitrification, the expression of $^{18}\epsilon_{\text{k,NO}}$ during subsequent NO_2^- oxidation can influence $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$. Moreover, applications of Eq. 1 and 2 commonly treat f_{ex} and the kinetic and equilibrium isotope effects as constant parameters.

145 Such formulations therefore cannot capture potentially transient changes in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ resulting from dynamic environmental conditions or changes in the relative rates of NH_4^+ and NO_2^- oxidation.

Table 1. Reported kinetic O isotope effects and the fraction of NO_2^- -water O isotope exchange (f_{ex}) during nitrification from soil, freshwater, seawater and culture incubation studies.

	$^{18}\epsilon_{\text{k,comp}}$ (‰) ^a	$^{18}\epsilon_{\text{k,H}_2\text{O},2}$ (‰)	$^{18}\epsilon_{\text{k,NO}}$ (‰)	f_{ex} (%)	Reference
Soil incubations	24.3 ± 1.0	4.5 ± 0.4	–	0–88	Hu et al., 2024; Snider et al., 2010
Freshwater incubations	8–27	14–23	-3.9 ± 0.3	74–78	Boshers et al., 2019
Seawater incubations	11–20	1–27	–	48–100	Buchwald et al., 2012
Cocultures	16–23	6–12	–	0–26	Buchwald et al., 2012
NH_3 oxidizing cultures	18–38	–	–	0–46 ^b	Casciotti et al., 2010; Nishizawa et al., 2016
NO_2^- oxidizing cultures	–	9–25	$-10 - -1$	0–3 ^b	Buchwald and Casciotti 2010

150 ^a $^{18}\epsilon_{\text{k,comp}} = ^{18}\epsilon_{\text{k,O}_2} + ^{18}\epsilon_{\text{k,H}_2\text{O},1}$

^b Reported O isotope exchange fractions reflect microbially mediated exchange only.

2.2 StorAge Selection Functions as Conceptual Transport Descriptors

StorAge Selection (SAS) functions provide a conceptual framework for representing hydrological transport regimes and for estimating the distribution of water ages within hydrological systems (Benettin et al., 2022). Here, water age is defined as the time elapsed since a water parcel entered the system as precipitation until it exits through discharge or evapotranspiration. Within the SAS framework, water stored in the system is ranked according to age (T), from youngest to oldest, and the evolution of this age-ranked storage – denoted as $S_T(T, t)$ – is tracked through time (t) as water enters and leaves the system (Fig. 2) (Botter et al., 2011; Harman, 2015):

$$\frac{\partial S_T(T, t)}{\partial t} = \sum_m Q_{\text{in}}^m(t) P_{\text{in}}^m(T, t) - \sum_n Q_{\text{out}}^n(t) P_{\text{out}}^n(T, t) - \frac{\partial S_T(T, t)}{\partial T} \quad (3)$$

160 where Q_{in}^m and Q_{out}^n denote the m th inflow to and n th outflow from the storage, respectively, and $P_{\text{in}}^m(T, t)$ and $P_{\text{out}}^n(T, t)$ represent their corresponding cumulative water age distributions. The final term on the r.h.s. of Eq. 3 represents the aging of water stored in the system, whereby all water parcels increase in age by one model time step as time progresses.

For precipitation inputs, water age is zero by definition. For each outflow leaving the storage, its cumulative water age distribution $P_{\text{out}}(T, t)$ depends on the cumulative age distribution of water residing in the storage, $P_S(T, t)$, and can be expressed as a function of the latter through a SAS function, Ω_{out} (van der Velde et al., 2012):

$$P_{\text{out}}(T, t) = \Omega_{\text{out}}(P_S(T, t)) \quad (4)$$

Thus, the SAS function characterizes how water of different ages within the storage is “selected” to contribute to the outflow.



In environmental applications, the SAS function is typically parameterized using a prescribed mathematical form (Benettin et al., 2022). Among several commonly used formulations, including gamma, beta, and uniform functions, the one-parameter power-law function has been widely adopted because of its simplicity (Benettin et al., 2017):

$$\Omega_{out}(P_S(T, t)) = P_S(T, t)^{k_{out}} \quad (5)$$

In Eq. 5, k_{out} is a shape parameter that determines the preference of an outflow to select water of different ages (Fig. 2). When $k_{out} < 1$, younger water is preferentially released, whereas $k_{out} > 1$ indicates preferential release of older water. The strength of age preference increases as k_{out} deviates further from 1. When $k_{out} = 1$, water is released uniformly across the age distribution of storage, corresponding to complete mixing.

These water selection behaviors, as reflected in the value of k_{out} , have been linked to physical transport regimes within the advection-dispersion framework (Benettin et al., 2013). Specifically, a pronounced preference for younger water ($k_{out} \ll 1$) is often associated with enhanced dispersion and the activation of preferential flow paths through soil macropores or permeable near-surface horizons (Schwemmler and Weiler, 2023; Türk et al., 2026). In contrast, a strong preference for older water ($k_{out} \gg 1$) may reflect more advective transport along longer flow paths or translatory water displacement through the soil matrix (Asadollahi et al., 2020; Schwemmler and Weiler, 2023). Values near $k_{out} \approx 1$ indicate a balanced interplay between advective and dispersive processes in the absence of strong preferential flow (Benettin et al., 2013). Thus, by varying k_{out} , the SAS framework can represent a continuum of transport behaviors spanning different flow path structures and water mixing regimes. This approach has been recently applied to investigate ecohydrological separation by assigning flux-specific SAS functions to evapotranspiration and streamflow (Knighton et al., 2019; Sprenger et al., 2022).

When $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ is used as a tracer to “tag” individual water parcels in storage, the isotopic composition of the outflow $\delta_{out}(t)$ can be readily computed from its water age distribution:

$$\delta_{out}(t) = \int_0^\infty \delta_s(T, t) p_{out}(T, t) dT \quad (6)$$

where $\delta_s(T, t)$ (‰) is the $\delta^{18}\text{O}$ of a water parcel with age T at time t within storage, $p_{out}(T, t)$ is the water age distribution of the outflow, obtained by differentiating its cumulative age distribution $P_{out}(T, t)$ with respect to age T . In this framework, $\delta_s(T, t)$ is governed by source inputs with potentially variable isotopic composition (e.g., precipitation) and by evaporative fractionation, which increases $\delta^{18}\text{O}$ in the remaining water. This evaporative enrichment can be incorporated within the age-ranked storage formulation to track evaporative losses from individual water parcels as they age and transit through the system (Benettin et al., 2017; Quéloz et al., 2015).

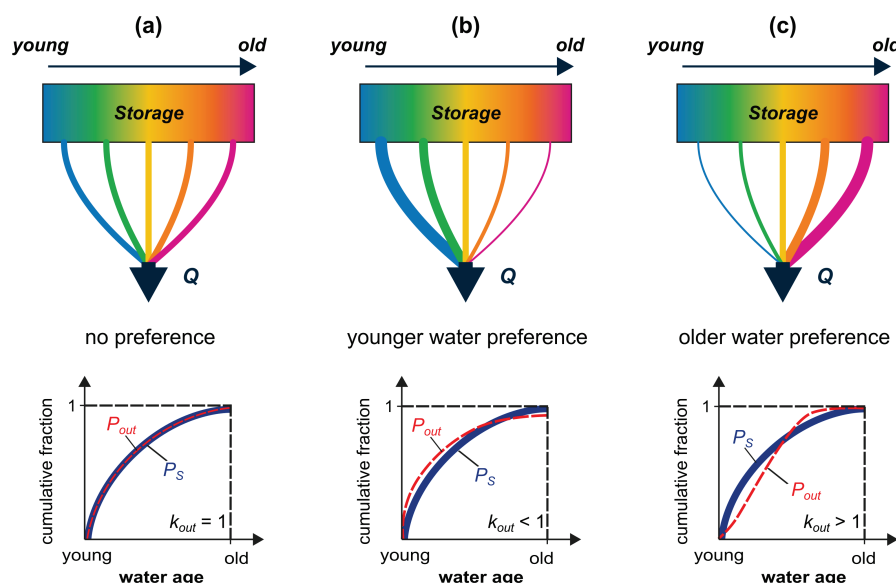


Figure 2. Illustration of three characteristic water storage selection behaviors, and the associated cumulative age distributions of storage (P_s , blue solid line) and outflow (P_{out} , red dashed line): (a) no preference ($k_{out} = 1$), (b) preference for younger water ($k_{out} < 1$), and (c) preference for older water ($k_{out} > 1$). The power-law exponent k_{out} controls how outflow samples storage of different ages: when $k_{out} = 1$, P_{out} coincides with P_s ; when $k_{out} < 1$, the outflow is biased toward younger water; when $k_{out} > 1$, the outflow is biased toward older water. Adapted from Yang et al. (2018).

3 Biogeochemical Quantification and Modeling of $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$

3.1 Soil Sample Collection and Characterization

Surface soil samples (0–30 cm) were collected from an agricultural field within the vicinity of the Upper Embarras River (UER) watershed in east-central Illinois, USA (Fig. S1). The region has a humid continental climate, with a long-term average annual precipitation of 1008 mm (NOAA, 2022). The dominant soil types at the study field are Drummer (fine-silty, mixed, superactive, mesic Typic Endoaquolls) silty clay loam and Flanagan (fine, smectitic, mesic Aquic Argiudolls) silt loam (USDA-NRCS, 2016). The field has been managed under a corn–soybean rotation for more than a decade, with N fertilizer applied during the corn phase every other year.

In May 2024, prior to fertilizer application, multiple soil cores (0–30 cm depth) were collected across the field using a soil corer and combined into a single composite sample. The composite soil was passed through a 4-mm sieve at field moisture, thoroughly homogenized, and stored at 4 °C until the incubation experiment. Selected physical and chemical properties of the composite soil are summarized in Table S1.

3.2 Laboratory Experimental Design

The incubation design largely followed established protocols for measuring potential soil nitrification rates (Taylor et al., 2010), while incorporating different ^{18}O -labeled water to quantify $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$. To initiate the incubation, 100 g of moist soil was mixed with 700 mL of 30 mM N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) buffer, adjusted to pH 7.2 with 10 M NaOH, in 2 L flasks. The flasks were then sealed with perforated phenolic caps wrapped in parafilm. TES was selected because it does not extract humic compounds from soil (Taylor et al., 2010), and pilot experiments confirmed that it provided sufficient buffering capacity to maintain the slurry pH at 7.1 ± 0.1 throughout the incubations.



Incubations were conducted at 7, 14, 21 (room temperature), 28, and 35 °C, spanning the typical annual soil temperature range in the study area (WARM, 2015). For the 21 °C treatment, soil slurries were amended with ammonium sulfate ((NH₄)₂SO₄) to establish five NH₄⁺ amendment levels: 0, 24.5, 49, 73.5, and 98 mg N kg⁻¹ soil, corresponding to increases in soil slurry NH₄⁺ concentration from 0 to 1 mM. The highest level approximated elevated NH₄⁺ conditions following fertilizer application at 250 kg N ha⁻¹, which is near the upper end of the typical range used in the UER region, whereas the lowest level represented background or unfertilized conditions. For the non-room-temperature treatments, two amendment levels (49 and 98 mg N kg⁻¹ soil) were used. Background NO₃⁻ concentrations in the soil were 9.6±1.1 mg N kg⁻¹ soil, substantially lower than the added NH₄⁺ concentrations. To quantify the dependence of δ¹⁸O_{NO₃,nit} on δ¹⁸O_{H₂O}, each temperature-NH₄⁺ treatment combination was conducted in triplicate using water with three distinct δ¹⁸O_{H₂O} values: -6.9‰ (unlabeled), 18.6‰, and 46.9‰. These were prepared by adding ¹⁸O-enriched water (10 atom % ¹⁸O; Cambridge Isotope Laboratory, USA) to deionized water. A summary of all treatment combinations is provided in Table 2.

Soil slurries were incubated aerobically on orbital shakers at 200 rpm. Temperature-specific treatments were maintained in a temperature-controlled Brunswick shaker, while the 21 °C treatment was incubated on a benchtop orbital shaker. The first samples were collected 30 minutes after incubation began. Subsequent sampling intervals ranged from 12 hours to 7 days, depending on temperature-driven differences in nitrification rates and NO₂⁻ accumulation. Incubation durations ranged from <2 weeks (21–35 °C) to ~3 months (7 °C), ensuring complete nitrification, as indicated by final NO₃⁻ concentrations exceeding added NH₄⁺ and the absence of detectable NO₂⁻ at the end of the incubation.

At each sampling event, flasks were briefly opened to allow gas exchange and sample collection. A 30 mL slurry aliquot was transferred to a centrifuge tube and centrifuged at 7000 rpm for 10 minutes. The supernatant was then filtered through 0.45 μm filters and stored at -20 °C until analyses. In total, 597 samples were collected during the incubation experiment.

Table 2. Summary of incubation treatments.

Treatments (total <i>n</i> = 39)	Temperature (°C)	NH ₄ ⁺ addition (mg N kg ⁻¹ soil)	Replicate 1 δ ¹⁸ O _{H₂O} (‰) ^a	Replicate 2 δ ¹⁸ O _{H₂O} (‰) ^a	Replicate 3 δ ¹⁸ O _{H₂O} (‰) ^a
1	35	98	-6.6	18.8	46.5
2	35	49	-6.6	18.6	47.0
3	28	98	-6.8	18.9	47.3
4	28	49	-7.0	18.9	47.1
5	21	98	-7.0	18.6	46.9
6	21	73.5	-6.9	18.6	46.7
7	21	49	-7.0	18.6	46.7
8	21	24.5	-6.9	18.6	47.1
9	21	0	-7.0	18.6	47.0
10	14	98	-7.0	18.5	47.0
11	14	49	-6.9	18.5	47.2
12	7	98	-7.0	18.4	46.7
13	7	49	-6.9	18.4	47.0

^a Values of δ¹⁸O_{H₂O} reflect mixing between the added water and endogenous soil water and therefore differ slightly among treatments.



3.3 Chemical and Isotopic Analyses

Chemical and isotopic analyses of soil water samples included measurements of NO_2^- and NO_3^- concentrations, as well as $\delta^{18}\text{O}_{\text{NO}_3}$ and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. Although the N isotopic composition of NO_3^- was also determined for all samples, those data are beyond the scope of the present study and will be reported separately; therefore, this paper focuses exclusively on $\delta^{18}\text{O}_{\text{NO}_3}$.

Concentrations of NO_2^- and NO_3^- were measured colorimetrically using a Hach Lachat QuikChem 8000 system. Isotope ratios of NO_3^- were determined following conversion to nitrous oxide (N_2O) via the denitrifier method (Weigand et al., 2016), with analysis on an Elementar Isoprime precisiON isotope ratio mass spectrometer. Prior to NO_3^- isotopic analysis, NO_2^- was removed using sulfamic acid (Granger and Sigman, 2009). International reference materials (USGS34, USGS35) and an internal NO_3^- working standard were analyzed alongside samples for calibration. During calibration, $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ of the incubation medium was explicitly accounted for to correct O isotope exchange between NO_2^- and water during conversion to N_2O (Casciotti et al., 2002). Analytical precision was $\pm 0.2\text{‰}$ for $\delta^{18}\text{O}_{\text{NO}_3}$.

Water isotope analyses were performed on the first slurry sample from each treatment to account for mixing between the incubation medium and endogenous soil water. Measurements were conducted using a Picarro L2130-I cavity ring-down spectrometer and calibrated against three international water standards (USGS46, USGS47, USGS48). Analytical precision for $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ was $\pm 0.1\text{‰}$.

3.4 Estimation of $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ and Oxygen Isotope Exchange

The $\delta^{18}\text{O}$ of nitrification-produced NO_3^- ($\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$) was estimated from the measured NO_3^- concentrations and $\delta^{18}\text{O}_{\text{NO}_3}$ values using an isotope mass balance (Hu et al., 2024):

$$\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}(t) = (\delta^{18}\text{O}_{\text{NO}_3}(t) \times [\text{NO}_3^-](t) - \delta^{18}\text{O}_{\text{NO}_3,\text{initial}} \times [\text{NO}_3^-]_{\text{initial}}) / ([\text{NO}_3^-](t) - [\text{NO}_3^-]_{\text{initial}}) \quad (7)$$

where $\delta^{18}\text{O}_{\text{NO}_3}(t)$ and $[\text{NO}_3^-](t)$ represent the isotopic composition and concentration of NO_3^- at time t , and $\delta^{18}\text{O}_{\text{NO}_3,\text{initial}}$ and $[\text{NO}_3^-]_{\text{initial}}$ are the corresponding values at the initial sampling time of each incubation.

The fraction of O isotope exchange between NO_2^- and water during nitrification (f_{ex}) was estimated using Eq. 2 and data from triplicate incubations spanning three distinct $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values. Specifically, f_{ex} was calculated from the slope of the linear regression between $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. Because Eq. 2 assumes complete oxidation of intermediate NO_2^- , only sampling times at which NO_2^- concentrations were below the detection limit ($0.7 \text{ mg N kg}^{-1} \text{ soil}$) were included in the regression analysis.

3.5 Dynamic Nitrification Model

A dynamic nitrification model was developed to simulate the temporal evolution of NO_2^- and NO_3^- concentrations and their O isotopic composition under varying temperature, NH_4^+ availability, and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. A complete description of the model derivation, governing equations, parameterization, and calibration procedure is provided in Supplementary Information Text S1.

Briefly, the model consists of two coupled components. The first is a biomass- and temperature-dependent reaction module based on Michaelis–Menten–Monod (MMM) kinetics that simulates N fluxes associated with mineralization (decomposition of soil organic N to NH_4^+), NH_4^+ oxidation, and NO_2^- oxidation (Maggi and Riley, 2009). The rate of each process is determined by a reaction-specific rate constant (k , h^{-1}), half-saturation concentration (K , mol L^{-1}), and enzyme abundance (E , mol L^{-1}). For each microbial functional group (organic N decomposers, NH_3 oxidizers, and NO_2^- oxidizers), enzyme abundance is assumed to be proportional to microbial biomass through an enzyme-to-biomass ratio (z). Microbial biomass increases through substrate utilization according to a biomass yield coefficient (Y , g mol^{-1} ; Monod, 1949) and decreases through mortality at rate m (h^{-1}). Consequently, the maximum specific growth rate under non-limiting substrate conditions (μ_{max} , h^{-1}) is estimated as $\mu_{\text{max}} = zkY$, and the corresponding net growth rate is $\mu_{\text{max}} - m$. The temperature dependence of k , K , Y , and m is represented using the near-activation



and differential activation (NADA) framework, which expresses these kinetic parameters as functions of temperature-dependent thermodynamic quantities (Maggi et al., 2018; Maggi and Riley, 2015) (see Supplementary Information Text S1 for more information).

The second component is a non-steady-state O isotope module coupled to the MMM framework. At each time step, the module resolves three sequential processes during nitrification: (1) NH_4^+ oxidation, including biologically mediated O exchange between NO_2^- and water; (2) NO_2^- oxidation to NO_3^- ; and (3) abiotic O exchange between NO_2^- and water (Fig. 1). During NH_4^+ oxidation, the $\delta^{18}\text{O}$ of newly produced NO_2^- is calculated using an isotope mass balance that accounts for the isotopic composition of water and O_2 , the fraction of biologically mediated O exchange ($f_{\text{ex,AO}}$), and the associated kinetic and equilibrium isotope effects ($^{18}\epsilon_{\text{k,comp}}$ and $^{18}\epsilon_{\text{eq}}$). As discussed below, the parameter $f_{\text{ex,AO}}$ is expressed as a linear function of instantaneous NO_2^- concentration, thereby linking the extent of biological O exchange to NO_2^- accumulation. During NO_2^- oxidation, preferential consumption of lighter or heavier NO_2^- isotopologues is represented using a Rayleigh formulation governed by the kinetic isotope effect $^{18}\epsilon_{\text{k,NO}}$, while incorporation of the third O atom from water into NO_3^- is associated with the kinetic isotope effect $^{18}\epsilon_{\text{k,H}_2\text{O},2}$. Finally, abiotic O isotope exchange between NO_2^- and water is represented as a temperature- and pH-dependent first-order equilibration process that drives the NO_2^- pool toward isotopic equilibrium with water (Buchwald and Casciotti, 2013). Following the treatment of the equilibrium isotope effect by Buchwald and Casciotti (2013), all kinetic isotope effects ($^{18}\epsilon_{\text{k,comp}}$, $^{18}\epsilon_{\text{k,H}_2\text{O},2}$, and $^{18}\epsilon_{\text{k,NO}}$) are parameterized as linear functions of temperature, with their slopes and intercepts estimated during model calibration.

Model calibration was performed sequentially using the PEST software with the Levenberg–Marquardt optimization algorithm (Doherty, 2004). First, the MMM module was calibrated collectively across all incubation treatments using observed NO_2^- and NO_3^- concentrations to constrain N transformation rates and associated biomass dynamics. The O isotopologue module was then coupled to the calibrated MMM module and optimized against measured $\delta^{18}\text{O}_{\text{NO}_3}$ values. This sequential strategy ensured that O isotope dynamics were constrained by independently calibrated N fluxes, thereby minimizing compensatory adjustments among N-flux and isotope parameters. All parameters subject to calibration for the MMM framework and the non-steady-state O isotopologue module are listed in Tables S2 and S3, respectively.

3.6 Scenario Analysis of Biogeochemical Controls on $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$

The optimized dynamic nitrification model was used to simulate $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ across gradients of temperature, NH_4^+ availability, and environmental $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. Two biogeochemical scenarios (B1 and B2; Table 3) were designed to guide this analysis. In scenario B1, $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ was simulated across a matrix of temperatures (7–35 °C, in 1 °C increments) and NH_4^+ additions (0–98 mg N kg⁻¹ soil, in 1 mg N kg⁻¹ soil increments), with reaction durations of 3, 7, 14, 30 and 90 days. The $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value was fixed at -6.9‰, corresponding to the non-labeled water treatment and representing background conditions in the study area.

In scenario B2, the same environmental matrix was applied with a fixed reaction duration of 14 days, while $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ was varied from -17.3‰ to 1.0‰ (0.2‰ increments), spanning the range of weekly precipitation $\delta^{18}\text{O}$ measured within the UER watershed during 2022–2024 (Miller et al., 2026). To quantify the dependence of $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ across all simulated environmental conditions, linear regressions of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ vs. $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ were fitted to the upper bound, lower bound, and median of simulated $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ values at each $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ grid point.



320 Table 3. Summary of biogeochemical and hydrological modeling scenarios.

Scenarios	Biogeochemical conditions	$\delta^{18}\text{O}_{\text{H}_2\text{O}}$ during nitrification	$\delta^{18}\text{O}_{\text{NO}_3, \text{nit}} - \delta^{18}\text{O}_{\text{H}_2\text{O}}$ relationship	Transport parameters
B1	Temperature: 7–35 °C NH_4^+ addition: 0–98 mg N kg ⁻¹ soil Reaction duration: 3, 7, 14, 30 and 90 days	Fixed at –6.9‰	Determined by model	-
B2	Temperature: 7–35 °C NH_4^+ addition: 0–98 mg N kg ⁻¹ soil Reaction duration: 14 days	–17.3‰ – –1‰	Determined by model	-
HB1	Constant daily NO_3^- production	Determined by model	Median fit from B2	k_E : 0.1–10 k_R : 0.1–10
HB2	Constant daily NO_3^- production in designated seasons	Determined by model	Upper–lower envelope from B2	k_E : 0.1–10 k_R : 0.1–10

4 Synthetic Transport Simulations of $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$

4.1 Hydrological Simulations

325 Variably saturated water flow through a 1-m soil column representative of the study site was simulated using HYDRUS-1D (Šimůnek et al., 2016) (Fig. 3). Because nitrification and evaporation occur primarily in the near-surface zone, the soil column was divided into two control volumes: a 30-cm topsoil layer and a 70-cm subsoil layer. This partitioning is also consistent with the incubation experiments, which were conducted using soil collected from the 0–30 cm depth interval. Both layers were represented as silty clay loam, consistent with site conditions, with hydraulic parameters estimated using the Rosetta pedotransfer functions (Schaap et al., 2001). The model was driven by meteorological observations from the UER region, including precipitation, air temperature, solar radiation, and relative humidity, for 2019–2022, spanning two complete corn–soybean rotations. All other soil and crop parameters were assigned their default HYDRUS-1D values, and no calibration was performed. Additional details on the HYDRUS-1D configuration are provided in Supplementary Information Text S2.

330 Simulated daily water fluxes between and within the topsoil and subsoil layers are shown in Fig. 3. In the topsoil, inflows include precipitation (P) and capillary rise (C), while outflows include evaporation (E), transpiration (T_1), and percolation (R). In the subsoil, inflow consists of percolation from the topsoil, and outflows include capillary rise, transpiration (T_2), and discharge (Q).
 335 The mean column discharge-to-precipitation ratio was 31% during the simulation period, closely matching the watershed-scale runoff ratio of 33% in the UER (Sha et al., 2025). Evaporation accounted for 47% of total evapotranspiration ($E/(E+T_1+T_2) = 0.47$). As expected for the regional climate, discharge at the bottom of the soil column occurred primarily from winter through early summer (December–June) (Fig. 3c), when the field was under bare fellow conditions. In contrast, evaporation and transpiration were highest during spring to early summer (April–June) and from midsummer through fall (July–October), respectively (Fig. 3c).

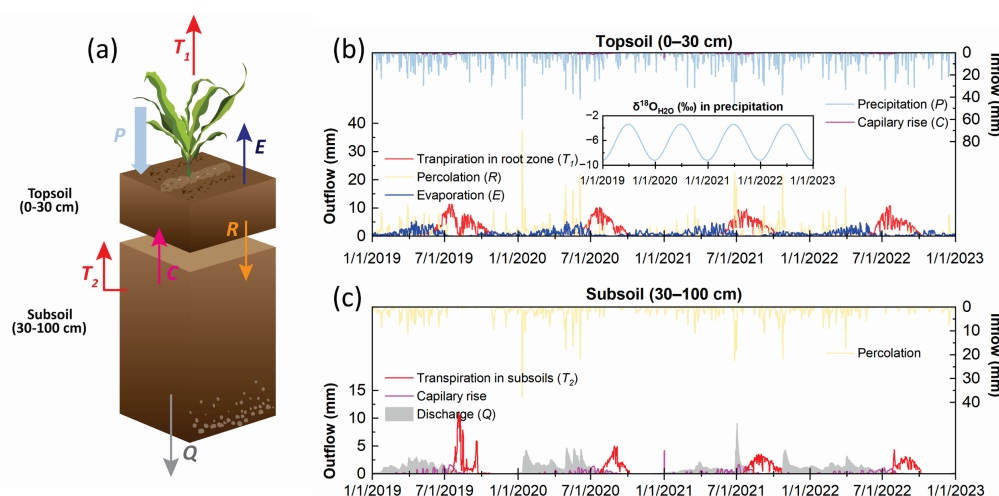


Figure 3. Conceptual hydrological model of a 1-m soil column with synthetic water fluxes. Panel (a) illustrates the model structure, while panels (b) and (c) show HYDRUS-1D-simulated water fluxes for the topsoil and subsoil layers, respectively. The inset in (b) presents the fitted sinusoidal seasonal cycle of precipitation $\delta^{18}\text{O}$ for the UER watershed.

4.2 Transport Modeling and SAS Functions

Simulated flow dynamics from HYDRUS-1D were used to drive daily water transport and $\delta^{18}\text{O}$ tracer dynamics within the SAS framework. Simulations were implemented using the open-source *trans-SAS* v1.0 package (Benettin and Bertuzzo, 2018b). Age-selective transport was parameterized for topsoil evaporation (E) and percolation (R) using the power-law SAS function (Eq. 5), with shape parameters k_E and k_R , respectively. Thus, although the magnitudes of the water fluxes were prescribed by the HYDRUS-1D simulations, varying k_E and k_R altered the age distributions of water contributing to evaporation and percolation and, consequently, the isotopic composition of these fluxes. All other outflows were assigned to follow complete mixing (i.e., no age preference), such that their cumulative age distributions and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values matched those of their respective source storage pools. This configuration isolates the effects of evaporative enrichment and the partitioning between evaporation and percolation as the primary controls on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ across storage pools and water fluxes.

For the SAS-based $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ simulations, seasonally varying precipitation $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ was prescribed as the tracer input using a sinusoidal function fitted to weekly precipitation $\delta^{18}\text{O}$ measurements at the UER watershed (Fig. 3b, inset; Miller et al., 2026). While transpiration was assumed to be non-fractionating, evaporative fractionation was implemented using the classic Craig-Gordon model (Craig and Gordon, 1965) as reformulated by Kuppel et al. (2018). This formulation of evaporative fractionation is coupled to the SAS framework to simulate temperature- and soil moisture-dependent isotopic enrichment of individual age-ranked water parcels within the topsoil. A detailed description of this coupling has been provided by our previous study (Miller et al., 2026) and is also summarized in Supplementary Information Text S3.

4.3 Scenario Analysis of $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ under Dynamic Transport Conditions

The $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ vs. $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ relationships derived from the dynamic nitrification model were incorporated into the synthetic transport model to simulate $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ in soil storage and column discharge. Two hydrological–biogeochemical scenario analyses (HB1 and HB2; Table 3) were conducted to evaluate (i) evaporative and hydrological controls on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$, and (ii) the variability of $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ under interacting hydrological and biogeochemical conditions.



In scenario HB1, hydrological effects on $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ were isolated from biogeochemical controls by prescribing a fixed $\delta^{18}\text{O}_{\text{H}_2\text{O}} - \delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ relationship based on the median fit derived from Scenario B2. At each time step, a unit mass of NO_3^- was produced in the topsoil and uniformly partitioned among age-ranked water parcels according to their fractional abundances. The $\delta^{18}\text{O}$ of newly formed NO_3^- within each parcel was then calculated from the parcel-specific $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ using the prescribed $\delta^{18}\text{O}_{\text{H}_2\text{O}} - \delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ relationship. Passive plant uptake associated with transpiration was included as a NO_3^- sink. Transport variability was represented through SAS functions by varying k_E and k_R , which were independently sampled from uniform distributions between 0.1 and 10, consistent with established ranges for the power-law SAS function (Asadollahi et al., 2020). A total of 1000 Monte Carlo realizations were conducted. To quantify the expression of evaporative enrichment, a baseline condition was also implemented in which evaporative fractionation was disabled and k_E and k_R were fixed at unity (i.e., complete mixing).

In scenario HB2, both hydrological and biogeochemical variability were considered simultaneously. Instead of prescribing the single median $\delta^{18}\text{O}_{\text{H}_2\text{O}} - \delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ relationship, each Monte Carlo realization randomly interpolated between the upper and lower bounding relationships generated under scenario B2. To investigate interactions between seasonal nitrification and hydrological partitioning, NO_3^- production was prescribed separately for winter (December-February), spring (March-May), summer (June-August), and fall (September-November), with NO_3^- production set to zero outside the target season. A year-round steady-state scenario was also simulated, in which NO_3^- production remained constant throughout the year. As in HB1, k_E and k_R were independently varied between 0.1 and 10 to represent a wide range of transport regimes. For each season, 1,000 Monte Carlo realizations were conducted.

5 Results

5.1 Soil Nitrification Experiment

5.1.1 Nitrogen concentrations

By the end of the incubations, cumulative NO_3^- production exceeded the amount of NH_4^+ added in all treatments (Fig. 4a–4e). Following NH_4^+ addition, NO_3^- concentrations initially increased slowly, particularly at lower temperatures, before entering a period of rapid accumulation. In all treatments except those at 35 °C, NO_3^- production slowed markedly shortly after cumulative production reached an amount equivalent to the added NH_4^+ . In contrast, the 21 °C treatment without NH_4^+ amendment exhibited a relatively steady increase in NO_3^- concentration, reaching 36 mg N kg⁻¹ soil by 300 h (Fig. 4c), indicating substantial NH_4^+ supply from native soil N mineralization. Across all temperature treatments, nitrification proceeded most rapidly at 28 °C. For example, under the 98 mg N kg⁻¹ soil NH_4^+ amendment, cumulative NO_3^- production reached the equivalent amount of added NH_4^+ after 140 h at 28 °C, compared with 177 h at 21 °C, 229 h at 35 °C, 482 h at 14 °C, and 1542 h at 7 °C (Fig. 4a–4e).

In most treatments, transient NO_2^- accumulation coincided with the period of rapid NO_3^- production (Fig. 4f–4j). Both the magnitude and persistence of NO_2^- accumulation were strongly influenced by temperature and NH_4^+ availability. At a given temperature, peak NO_2^- concentrations increased progressively with increasing NH_4^+ addition. For example, at 21 °C, peak NO_2^- concentration increased from below the detection limit to 21.9 ± 2.1 (1 SD) mg N kg⁻¹ soil as NH_4^+ addition increased from 0 to 98 mg N kg⁻¹ soil (Fig. 4h). In contrast, the temperature dependence of peak NO_2^- accumulation was nonmonotonic, with the highest concentrations generally observed at intermediate temperatures (14–28 °C; Fig. 4f–4j). However, the persistence of NO_2^- increased markedly with decreasing temperature. Detectable NO_2^- persisted for approximately 1600 h at 7 °C (Fig. 4j), compared with only ~150 h at 28 °C (Fig. 4g).

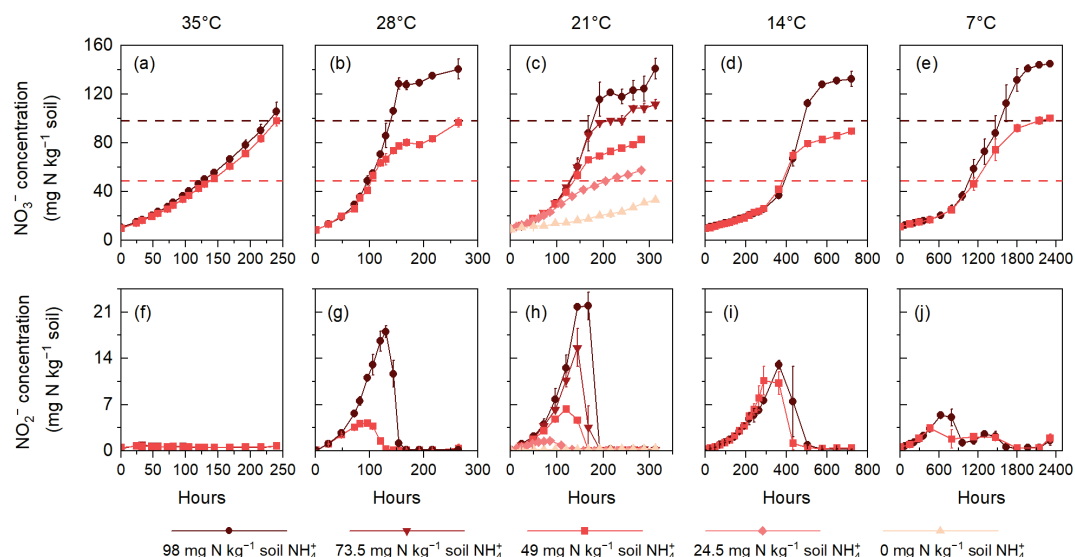


Figure 4. Concentrations of NO_3^- and NO_2^- during soil slurry incubations. Symbols with different colors denote the levels of NH_4^+ addition. The dashed horizontal lines in (a)–(e) indicate NH_4^+ addition levels of 98 mg N kg⁻¹ soil and 49 mg N kg⁻¹ soil. Error bars represent mean $\pm$ SD across replicates.

5.1.2 Oxygen isotopes

Across all treatments, measured $\delta^{18}\text{O}_{\text{NO}_3}$ values progressively approached the $\delta^{18}\text{O}$ of the incubation water as nitrification proceeded (Fig. 5a–5e). In treatments amended with –6.9‰ water, $\delta^{18}\text{O}_{\text{NO}_3}$ decreased from approximately 0‰ and gradually converged toward $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. In contrast, under 18.6‰ and 46.9‰ water, $\delta^{18}\text{O}_{\text{NO}_3}$ increased during nitrification and subsequently stabilized at values below the $\delta^{18}\text{O}$ of the incubation water. Moreover, the stabilized $\delta^{18}\text{O}_{\text{NO}_3}$ values increased with NH_4^+ addition. For example, at 21 °C with 46.9‰ water, stabilized $\delta^{18}\text{O}_{\text{NO}_3}$ values increased from 22‰ with no NH_4^+ amendment to 36‰ under NH_4^+ addition of 98 mg N kg⁻¹ soil (Fig. 5c).

Estimated $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ showed a similar separation among the three water treatments, converging toward $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ under the –6.9‰ water treatment but exhibiting strong sensitivity to NH_4^+ availability under the 18.6‰ and 46.9‰ water treatments (Fig. 5f–5j).

Unlike the measured $\delta^{18}\text{O}_{\text{NO}_3}$, estimated $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ exhibited transient maxima during periods of rapid NO_3^- production, particularly at lower temperatures and higher NH_4^+ additions. These transient maxima coincided closely with periods of peak NO_2^- accumulation (Fig. 4f–4j).

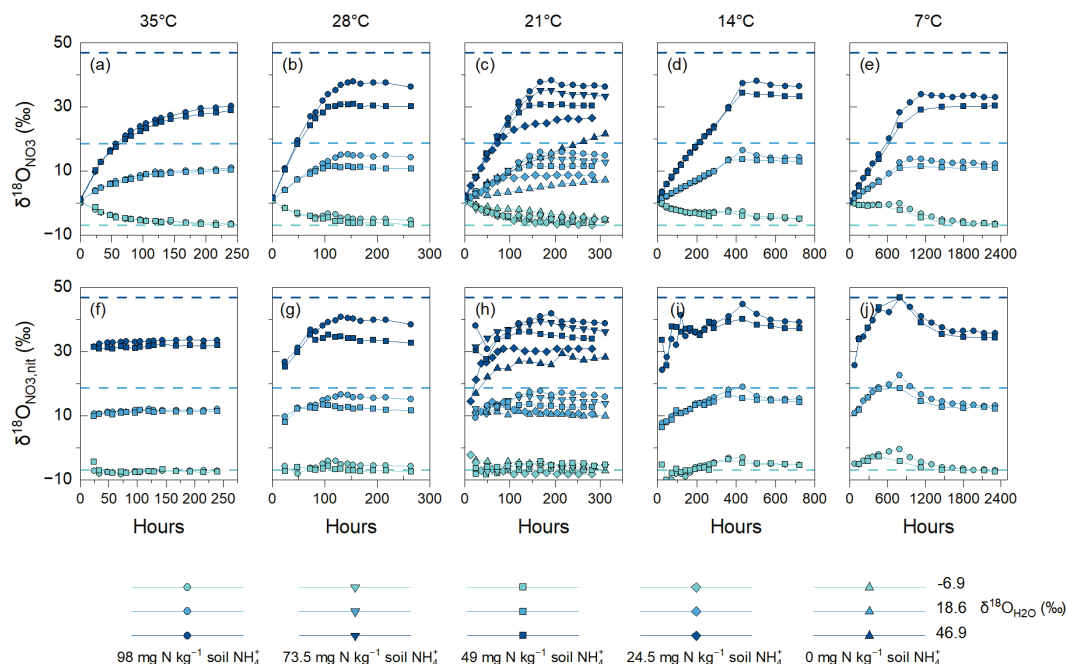


Figure 5. Temporal evolution of $\delta^{18}\text{O}_{\text{NO}_3}$ and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ values during soil slurry incubations. Colors represent the three $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ treatments (-6.9‰ , 18.6‰ , and 46.9‰), which are indicated by horizontal dashed lines in each panel. Symbols denote the five levels of NH_4^+ amendment.

Using $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ estimates from the three $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ treatments, linear regressions of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ against $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ were performed at the first sampling time when NO_2^- concentrations declined below the detection limit, thereby capturing the time-integrated impact of NO_2^- accumulation on $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$. For treatments exhibiting negligible NO_2^- accumulation (35 °C and the 21 °C treatment without NH_4^+ addition), regressions were based on the final sampling time. Strong linear relationships between $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ were observed across all treatments ($R^2 = 0.999$, $p \leq 0.01$; Fig. 6). The regression slopes yielded f_{ex} values ranging from 0 to 0.63 across the temperature and NH_4^+ gradients (Fig. 6). Estimated f_{ex} was strongly and positively correlated with the magnitude of peak NO_2^- accumulation (Fig. S2). For example, under the 98 mg N kg⁻¹ soil NH_4^+ amendment, f_{ex} reached its highest value at 21 °C (0.63; Fig. 6c), where peak NO_2^- accumulation was greatest, and its lowest value at 35 °C (0.30; Fig. 6a), where NO_2^- accumulation was negligible. At a given temperature, f_{ex} likewise increased with NH_4^+ addition, consistent with the greater transient NO_2^- accumulation observed under higher substrate availability.

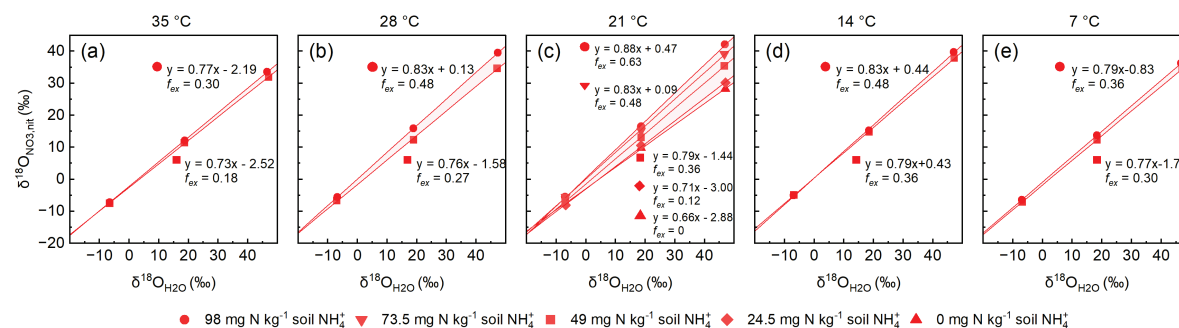


Figure 6. Dependence of $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ under different temperatures and NH_4^+ additions. Regressions are based on measurements taken at the sampling time when NO_2^- concentrations first declined to the detection limit, or at the end of the incubation if no appreciable



NO_2^- accumulated. Symbols denote different NH_4^+ amendment levels. The equations in each panel represent the linear fits for each NH_4^+ level at a given temperature. The O exchange fraction f_{ex} is calculated as $f_{ex} = 3 \times \text{slope} - 2$, following Eq. 2.

5.2 Simulating Nitrification Using the Dynamic Model

Calibrated optimal parameter values for the MMM and O isotope modules are summarized in Tables S2 and S3, respectively.

Overall model performance was strong, with correlation coefficients exceeding 0.40, 0.97, and 0.99 for NO_2^- , NO_3^- , and $\delta^{18}\text{O}_{\text{NO}_3}$, respectively (Fig. S3–S7). Model–data mismatches were primarily limited to periods of low NO_2^- concentrations (e.g., $<1 \text{ mg N kg}^{-1}$ soil; see Fig. S7). These discrepancies likely reflect both the transient nature of NO_2^- dynamics and the relatively high analytical detection limit (0.7 mg N kg^{-1} soil), which can obscure small concentration changes near the detection threshold.

The calibrated MMM module revealed distinct temperature responses of the kinetic parameters governing NH_4^+ and NO_2^- oxidation (Fig. 7). For both processes, the reaction rate constants (k) and half-saturation constants (K) exhibited nonmonotonic temperature dependence, peaking at 26–27 °C (Fig. 7a,b). The rate constant for NO_2^- oxidation was substantially more temperature-sensitive and consistently exceeded that of NH_4^+ oxidation, particularly at higher temperatures (Fig. 7a). In contrast, biomass yield coefficients (Y) exhibited U-shaped temperature responses and were consistently greater for NH_3 oxidizers than for NO_2^- oxidizers (Fig. 7c). As a result, combining k and Y , the maximum specific growth rate (μ_{max}) increased monotonically with temperature and was similar for the two functional groups (Fig. 7d). Because the mortality rate (m) also increased with temperature (Fig. 7e), the net specific growth rate ($\mu_{\text{max}} - m$) displayed a nonmonotonic response, reaching maxima at 29–30 °C (Fig. 7f). Net growth rates were higher for NH_3 oxidizers between approximately 19 and 33 °C, whereas NO_2^- oxidizers exhibited higher net growth rates outside this temperature range.

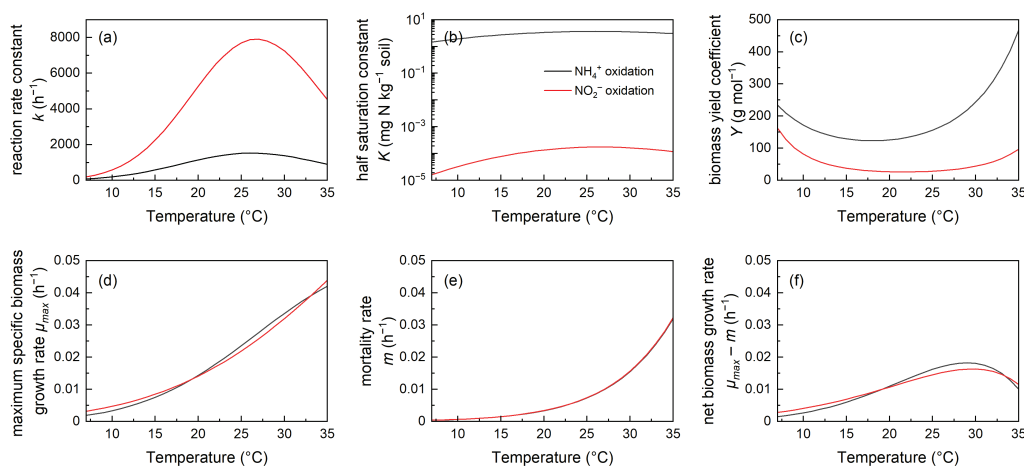


Figure 7. Temperature dependence of kinetic parameters (k , K , Y , μ_{max} , m , and $\mu_{\text{max}} - m$) derived from the optimized MMM model.

The O isotope effects derived from the calibrated isotope module are shown in Fig. 8. Normal kinetic isotope effects were obtained for $^{18}\epsilon_{k,\text{comp}}$ (31.3–36.6‰) and $^{18}\epsilon_{k,\text{H}_2\text{O},2}$ (1.9–4.4‰), whereas $^{18}\epsilon_{k,\text{NO}}$ exhibited an inverse isotope effect, ranging from –5.5 to –1.3‰. These modeled values fall within the ranges reported from previous laboratory experiments (Table 1). Overall, all three isotope effects showed relatively weak temperature dependence, varying by less than 5.3‰ across the investigated temperature range (7–35 °C) (Fig. 8; linear regression slopes and intercepts are provided in Table S3). Specifically, $^{18}\epsilon_{k,\text{comp}}$ increased with temperature, whereas $^{18}\epsilon_{k,\text{H}_2\text{O},2}$ decreased, and $^{18}\epsilon_{k,\text{NO}}$ became less negative, indicating reduced kinetic fractionation at higher temperature.

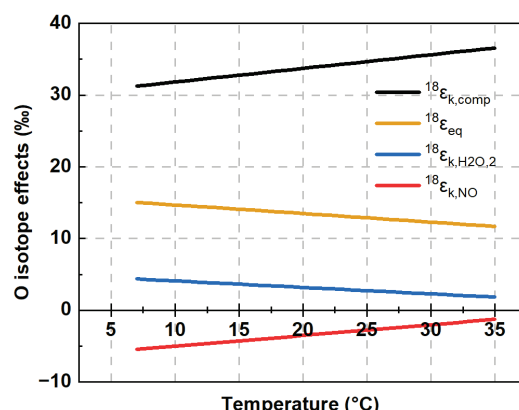


Figure 8. Temperature-dependent kinetic O isotope effects estimated from the calibrated O isotope module. Equilibrium isotope effects ($^{18}\epsilon_{eq}$) derived from Buchwald and Casciotti (2013) are also shown for comparison.

The optimized dynamic model identified a linear relationship between biologically mediated O exchange during NH_4^+ oxidation ($f_{ex,AO}$) and instantaneous NO_2^- concentration: $f_{ex,AO} = 0.0272 \times [NO_2^-] + 0.13$, where $[NO_2^-]$ is expressed in $mg\ N\ kg^{-1}$ soil (Table S3). Accordingly, simulated daily $f_{ex,AO}$ closely tracked NO_2^- dynamics, ranging from approximately 0.13 at low NO_2^- concentrations to as high as 0.76 during periods of peak NO_2^- accumulation (Fig. S8). To facilitate comparison with the regression-derived f_{ex} , daily $f_{ex,AO}$ values were weighted by the corresponding NH_4^+ oxidation rates and integrated from the start of the incubation to each sampling time used to estimate f_{ex} . The resulting time-integrated $f_{ex,AO}$ reproduced the temperature- and substrate-dependent patterns of the regression-derived f_{ex} (Fig. S8). For example, under the $49\ mg\ N\ kg^{-1}$ soil NH_4^+ amendment, model-derived $f_{ex,AO}$ values were 0.13, 0.21, 0.26, 0.29, and 0.16 at 35, 28, 21, 14, and 7 °C, respectively (Fig. S8), compared with regression-derived f_{ex} estimates of 0.18, 0.27, 0.36, 0.36, and 0.30 (Fig. 6). Excluding the 21 °C treatment without NH_4^+ addition, for which the regression-derived f_{ex} was zero, the ratio of model-derived $f_{ex,AO}$ to regression-derived f_{ex} averaged 0.78 ± 0.25 (1 SD) across all treatments.

5.3 Modeled Response of $\delta^{18}O_{NO_3,nit}$ to Temperature, NH_4^+ availability, and Environmental $\delta^{18}O_{H_2O}$

In scenario B1, with $\delta^{18}O_{H_2O}$ fixed at -6.9‰ , simulated $\delta^{18}O_{NO_3,nit}$ varied systematically across temperatures, NH_4^+ availability, and reaction times (Fig. 9). The difference between $\delta^{18}O_{NO_3,nit}$ and $\delta^{18}O_{H_2O}$ ($\delta^{18}O_{NO_3,nit} - \delta^{18}O_{H_2O}$) ranged from -1.4 to 3.1‰ , with the largest positive deviations occurring during the intermediate stages of nitrification (7–30 days; Fig. 9a–9e). During this period, the response surface exhibited a bimodal pattern: elevated $\delta^{18}O_{NO_3,nit} - \delta^{18}O_{H_2O}$ values occurred either at low temperatures across all NH_4^+ addition levels or at intermediate temperatures (approximately 15–30 °C) under high NH_4^+ availability. In contrast, the most negative differences consistently occurred at the highest temperatures ($\geq 33\text{ °C}$) under low NH_4^+ availability.

Analysis of cumulative NH_4^+ and NO_2^- oxidation revealed that these isotopic differences were closely associated with the degree of kinetic coupling between the two nitrification steps (Fig. 9f–9j). During the early and intermediate stages of nitrification (3–14 d), lower-to-intermediate temperatures and/or higher NH_4^+ availability promoted substantial decoupling between NH_4^+ and NO_2^- oxidation, resulting in NO_2^- accumulation and larger positive $\delta^{18}O_{NO_3,nit} - \delta^{18}O_{H_2O}$ values. By day 30, NH_4^+ and NO_2^- oxidation had largely returned to kinetic coupling under most simulated conditions, with persistent decoupling occurring only at the lowest temperatures (Fig. 9d,i). By day 90, complete kinetic coupling was achieved across all simulated conditions, and the range of $\delta^{18}O_{NO_3,nit} - \delta^{18}O_{H_2O}$ had narrowed to between -1.4 and 1.7‰ (Fig. 9e,j).

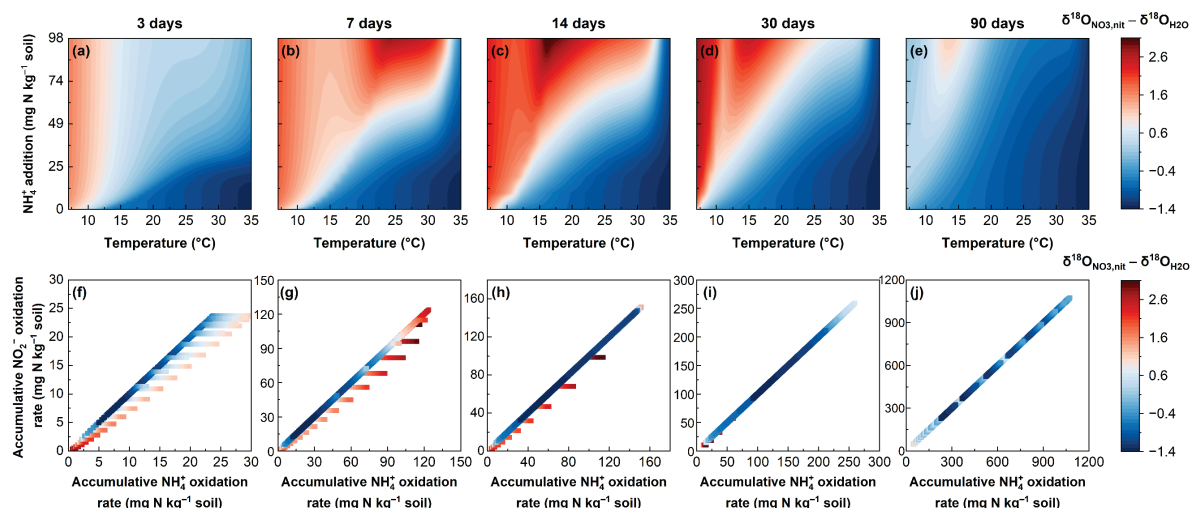


Figure 9. Predicted $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}} - \delta^{18}\text{O}_{\text{H}_2\text{O}}$ values under varying temperature, NH_4^+ availability, and reaction time scenarios (3, 7, 14, 30, and 90 days), with $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ fixed at -6.9‰ (a–e). Panels (f–j) show the relationship between accumulative NH_4^+ oxidation rates and NO_3^- oxidation rates at the reaction times.

In scenario B2, with reaction time fixed at 14 days, predicted $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ varied linearly with $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ across the entire range of temperature and NH_4^+ availability (Fig. 10). The resulting $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ vs. $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ relationship was bounded by two linear regressions: an upper bound of $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}} = 0.81 \times \delta^{18}\text{O}_{\text{H}_2\text{O}} + 1.86$ and a lower bound of $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}} = 0.71 \times \delta^{18}\text{O}_{\text{H}_2\text{O}} - 3.31$ (Fig. 10). The vertical separation between these bounds ranged from 3.4 to 5.3‰ across the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ gradient, representing the variability in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ driven by the imposed biogeochemical conditions at a given $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. The regression through the median simulated values was $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}} = 0.76 \times \delta^{18}\text{O}_{\text{H}_2\text{O}} - 0.73$. These simulated relationships fell well below predictions based on the conventional 2:1 O atom incorporation assumption, while largely encompassing the 1:1 relationship between $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ when $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ ranged approximately from -10‰ to 1‰ (Fig. 10). As $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ became increasingly negative, simulated $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ shifted progressively above the 1:1 line.

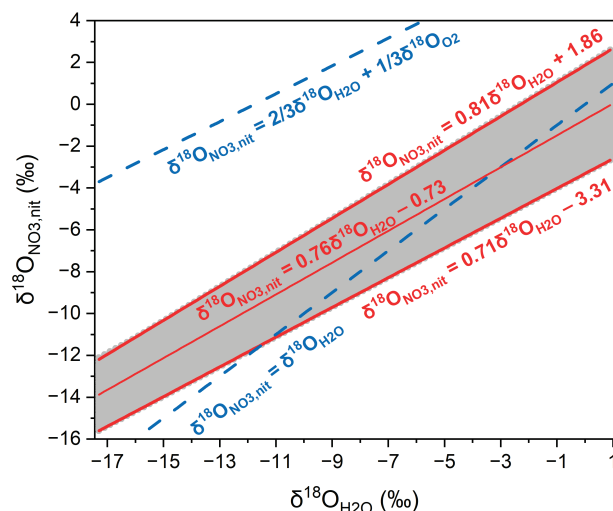


Figure 10. Predicted $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ values (gray area) as a function of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ (-17.3‰ to 1‰) under varying temperature ($7\text{--}35\text{ °C}$) and NH_4^+ addition levels ($0\text{--}98\text{ mg N kg}^{-1}\text{ soil}$) at 14 days. Red solid lines denote the upper bound, lower bound, and median of simulated $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ values as a function of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ (equations shown). Blue dashed lines represent $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ values inferred directly from $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ using commonly applied stoichiometric relationships (equations shown).



5.4 Expression of Evaporative Enrichment in $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$

In scenario HB1, under the baseline assumption of complete mixing without evaporative fractionation, $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in the topsoil exhibited a pronounced seasonal cycle driven by precipitation inputs, reaching values as high as -4‰ in summer and declining to approximately -8‰ in winter (Fig. 11a). A similar seasonal pattern was evident for $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in column discharge, although its amplitude was significantly attenuated (Fig. 11c). Seasonal variability in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ was also apparent in both the topsoil and column discharge (Fig. 11e,g). However, compared with $\delta^{18}\text{O}_{\text{H}_2\text{O}}$, the seasonal variation in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ was considerably dampened and exhibited a lag of approximately two to three months, particularly for $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in column discharge (Fig. 11g).

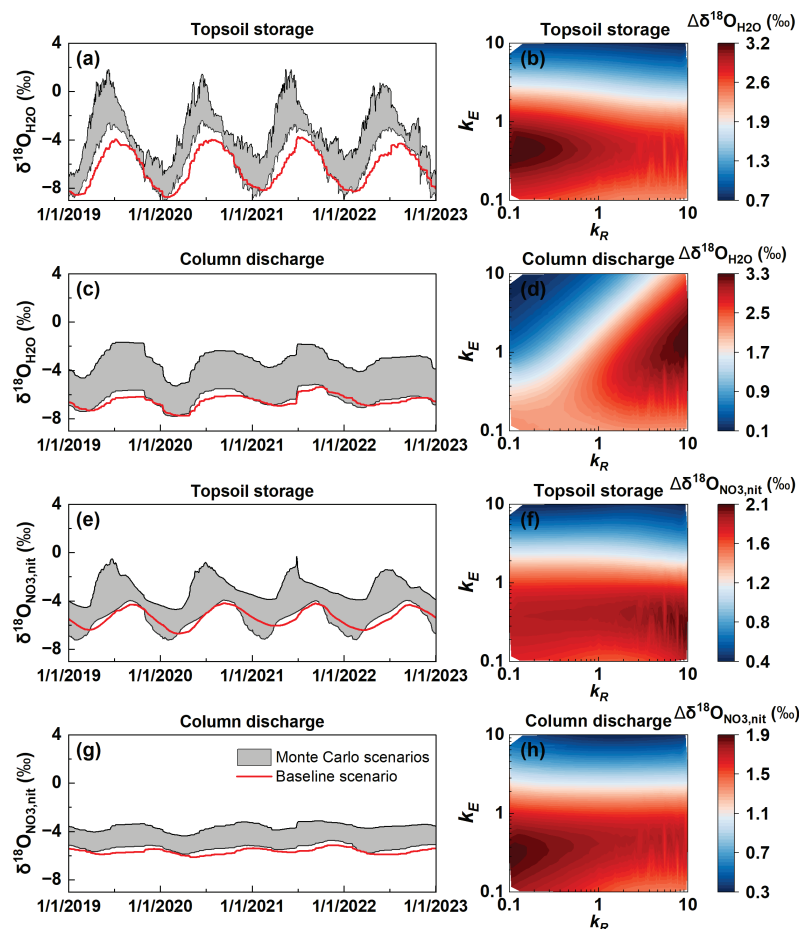


Figure 11. Temporal dynamics of simulated $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in topsoil storage (a) and column discharge (c), and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in topsoil storage (e) and column discharge (g). Red lines denote the baseline scenario, and gray shading represents the range derived from Monte Carlo scenarios in which k_E and k_R were varied. Deviations of mass-weighted $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ (b, d) and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ (f, h) from the baseline scenario ($\Delta\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\Delta\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$) are shown across the $k_E - k_R$ parameter space.

Introducing evaporative fractionation increased both $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ relative to the baseline scenario across the full range of simulated transport regimes (Fig. 11a,c,e,g). The effect was strongest in the topsoil during late spring and early summer, when evaporation increased daily $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ values by up to approximately 8‰ and 4‰ , respectively (Fig. 11a,e). In contrast, the evaporative enrichment was substantially attenuated in column discharge where mixing and transport dampened the seasonal variability of both isotopic signals (Fig. 11c,g).



To isolate the influence of hydrologic transport from seasonal variability in precipitation $\delta^{18}\text{O}$, we calculated annual mass-weighted mean $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ values for the topsoil and column discharge and expressed their differences from the baseline scenario as $\Delta\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\Delta\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$, respectively. Evaporation increased annual mean $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ by 0.7–3.2‰ in the topsoil and by 0.1–3.3‰ in column discharge across the simulated transport regimes (Fig. 11b,d). In the topsoil, $\Delta\delta^{18}\text{O}_{\text{H}_2\text{O}}$ was controlled primarily by the age preference of evaporation (k_E) and showed little sensitivity to the age preference of percolation (k_R) (Fig. 11b). Enrichment was greatest when evaporation preferentially removed relatively young water ($k_E \approx 0.5$) and progressively weakened as evaporation increasingly drew on older water ($k_E > 1$). In column discharge, however, $\Delta\delta^{18}\text{O}_{\text{H}_2\text{O}}$ reflected the combined effects of k_E and k_R (Fig. 11d). The strongest enrichment occurred when evaporation showed little age preference ($k_E \approx 1$) while percolation preferentially removed the oldest water from the topsoil ($k_R \gg 1$). Conversely, enrichment approached zero when evaporation preferentially removed the oldest water ($k_E \gg 1$) and percolation preferentially exported the youngest water ($k_R \ll 1$). Evaporation also increased the annual mass-weighted mean $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ by 0.4–2.1‰ in the topsoil and by 0.3–1.9‰ in column discharge (Fig. 11f,h). In the topsoil, $\Delta\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ showed a similar dependence on k_E as $\Delta\delta^{18}\text{O}_{\text{H}_2\text{O}}$, with the greatest enrichment occurring when evaporation preferentially removed relatively young water (Fig. 11f). In contrast to $\delta^{18}\text{O}_{\text{H}_2\text{O}}$, however, $\Delta\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in column discharge closely tracked the enrichment established in topsoil storage, remained primarily sensitive to k_E , and showed little sensitivity to k_R (Fig. 11h).

To evaluate whether $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in column discharge can be predicted by the corresponding $\delta^{18}\text{O}_{\text{H}_2\text{O}}$, we used the simulated $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in column discharge with the prescribed $\delta^{18}\text{O}_{\text{H}_2\text{O}}-\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ relationship and calculated the prediction bias as the predicted $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ minus the true $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ simulated by the synthetic transport model. Daily prediction biases ranged from approximately –2 to 2‰ across the simulated transport regimes (Fig. 12a). The bias was generally more positive during summer and fall and more negative during winter and spring. When integrated over the entire simulation period using mass-weighted means, prediction bias ranged from –1.3 to 1.4‰ and was primarily controlled by k_R with little sensitivity to k_E (Fig. 12b). Specifically, using column discharge $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ to predict $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ resulted in an underestimation when percolation preferentially exported younger water ($k_R < 1$) but an overestimation when percolation preferentially exported older water ($k_R > 1$).

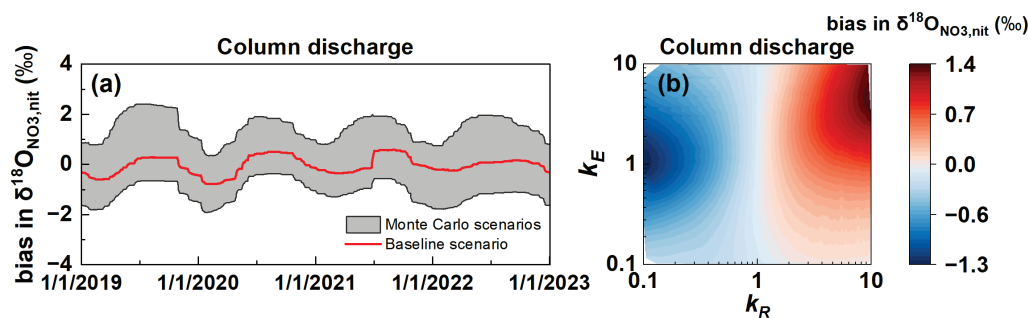


Figure 12. (a) Daily prediction bias in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ of column discharge under the baseline scenario (red line) and across Monte Carlo scenarios (gray shading). (b) Prediction bias in mass-weighted mean $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ of column discharge across the k_E-k_R parameter space.

5.5 Variability of $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ under Covarying Hydrological and Biogeochemical Conditions

Results from scenario HB2, in which biogeochemical variability, evaporative enrichment, and age-selective transport were considered simultaneously, are shown in Fig. 13. Despite the wide range of prescribed k_E and k_R values, mass-weighted mean $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in column discharge varied over a relatively narrow range (–6.7 to –3.5‰; Fig. 13a). Under year-round nitrification, the corresponding mass-weighted mean $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ ranged from –7.5 to –1.7‰, broadly spanning a range similar to that of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. In contrast, restricting nitrification to individual seasons produced pronounced shifts in simulated $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ (Fig. 13a). The highest



values occurred when nitrification was confined to spring (-6.9 to 0.0‰) or summer (-5.3 to 0.5‰), whereas the lowest values occurred under winter-only nitrification (-8.9 to -2.9‰). Simulated $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ under fall-only nitrification closely resembled that under year-round nitrification.

To evaluate whether $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in column discharge could be predicted from the corresponding $\delta^{18}\text{O}_{\text{H}_2\text{O}}$, we again calculated the prediction bias for each nitrification timing scenario. Rather than using the actual $\delta^{18}\text{O}_{\text{H}_2\text{O}}-\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ relationship prescribed in each Monte Carlo realization, we used the median $\delta^{18}\text{O}_{\text{H}_2\text{O}}-\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ relationship derived from scenario B2 to predict $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$. Applying a fixed relationship reflects the practical constraint that the specific $\delta^{18}\text{O}_{\text{H}_2\text{O}}-\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ relationship within nitrification source zones is generally unknown *a priori* in field applications.

Under year-round nitrification, prediction bias ranged from -3.3 to 3.2‰ across the 1000 Monte Carlo realizations and was approximately normally distributed, with a mean of 0‰ and a standard deviation of 1.4‰ (Fig. 13f). Restricting nitrification to individual seasons produced a similar overall spread of prediction biases (approximately $6\text{--}7\text{‰}$) but shifted the distribution substantially depending on the timing of nitrification. Spring- and summer-only nitrification yielded mean negative biases of -1.2‰ and -2.2‰ , respectively (Fig. 13b,c), whereas winter-only nitrification produced a positive mean bias of 1.4‰ (Fig. 13e).

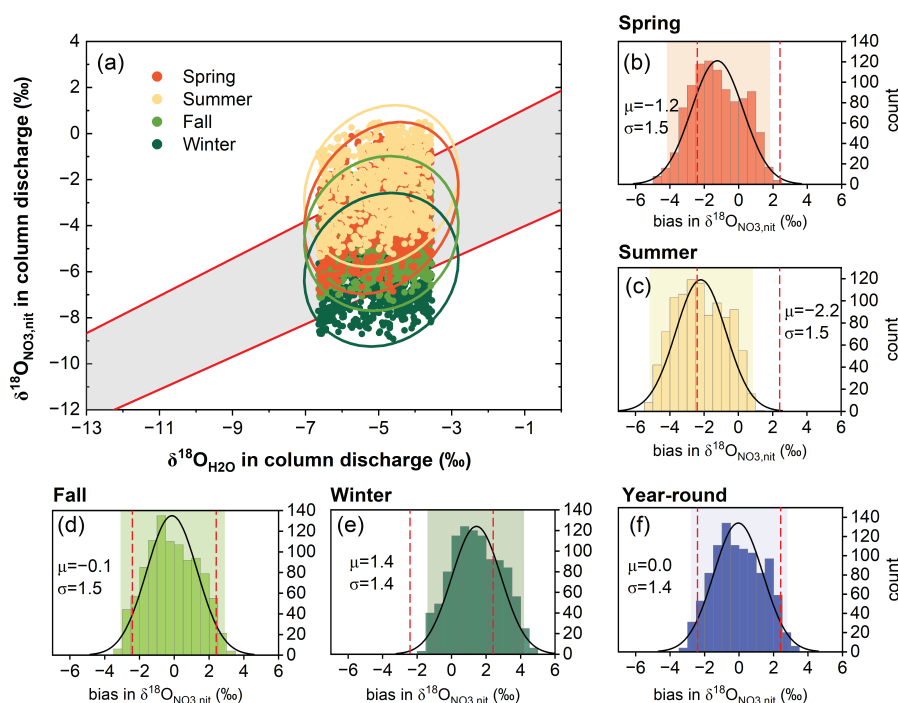


Figure 13. (a) Simulated mass-weighted $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ and corresponding $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in column discharge across the full range of biogeochemical conditions and hydrological transport scenarios, with NO_3^- production prescribed in designated seasons (colors). Fitted ovals represent 95% prediction ellipses, and the gray envelope bounded by red lines denotes the biogeochemical $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ range adopted from Fig. 10. Distributions of prediction bias in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ under seasonal (b-e) and year-round (f) nitrification scenarios. Black curves are fitted normal distributions (μ and σ indicated in each panel) and shaded color bands span $\mu \pm 2\sigma$. Red dashed lines indicate the range of prediction bias resulting from uncertainty in the biogeochemical $\delta^{18}\text{O}_{\text{H}_2\text{O}}-\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ relationship alone.

6 Discussion

6.1 Nitrite Accumulation and Oxygen Isotope Exchange

Our incubation and modeling results revealed substantial short-term variability in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$, particularly following high NH_4^+ amendment and at low to intermediate temperatures (Fig. 5f–5j and 9a–9e). These conditions coincided with pronounced NO_2^-



accumulation (Fig. 4f–4j and 9f–9j), which was positively correlated with time-integrated f_{ex} across treatments (Fig. 6 and S2).

This association supports a mechanistic link between NO_2^- accumulation and enhanced O isotope exchange between NO_2^- and water. Because greater O isotope exchange promotes the expression of equilibrium isotope fractionation, it increases $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ relative to $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and thus provides an explanation for the transient $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ maxima observed during nitrification (Fig. 5f–5j). Transient NO_2^- accumulation is common in agricultural soils, particularly following manure or NH_3 -based fertilizer applications (Jones and Hedlin, 1970; Maharjan and Venterea, 2013; Giguere et al., 2018). It has been primarily attributed to increased NH_4^+ availability and/or NH_3 inhibition of NO_2^- oxidation, which temporarily increase NH_4^+ oxidation rates relative to NO_2^- oxidation (Smith et al., 1997). Temperature can further influence NO_2^- accumulation because NH_4^+ and NO_2^- oxidation often exhibit different temperature sensitivities; however, the direction of this effect varies among studies, with enhanced NO_2^- accumulation reported under low ($\leq 10^\circ\text{C}$) (Venterea et al., 2020), intermediate ($20\text{--}30^\circ\text{C}$) (Schaefer and Hollibaugh, 2017), and high ($>30^\circ\text{C}$) (Duan et al., 2020) temperatures.

In our study, nitrification proceeded most rapidly at 28°C (Fig. 4a–4e). Consistent with this result, the calibrated MMM model predicted that reaction rate constants and net biomass growth rates for both NH_4^+ and NO_2^- oxidation peaked at approximately $26\text{--}30^\circ\text{C}$ (Fig. 7). This range is consistent with reported optimum temperatures for AOB and NOB ($25\text{--}35^\circ\text{C}$), although it is near the lower end of the reported range for AOA ($30\text{--}40^\circ\text{C}$) (Ouyang et al., 2017; Taylor et al., 2017; Taylor et al., 2019). Despite these broadly similar temperature responses between NH_4^+ and NO_2^- oxidation, pronounced NO_2^- accumulation occurred at low to intermediate temperatures. Analysis of the optimized MMM module indicated that this accumulation arose from several interacting constraints, including low initial NO_2^- oxidizer biomass (Tables S2 and S3), a steeper decline in the NO_2^- oxidation rate constant than in the NH_4^+ oxidation rate constant at lower temperatures (Fig. 7a), and slightly greater net biomass growth of NH_3 oxidizers than NO_2^- oxidizers at intermediate temperatures (Fig. 7f). A detailed discussion of these model-derived mechanisms is provided in Supplementary Information Text S4. More broadly, transient NO_2^- accumulation may reflect the relatively low energy yield of NO_2^- oxidation and the mixotrophic strategies of soil NOB, which help them persist under frequent NO_2^- limitation (Tappe et al., 1999; Ni et al., 2008). For example, Taylor et al. (2019) showed across five agricultural soils that NO_2^- oxidizers required de novo protein synthesis to restore metabolic activity following NH_4^+ amendment. This suggests that transient NO_2^- accumulation may partly reflect a lag in NOB physiological acclimation, during which enzyme production must increase before NO_2^- oxidation can sufficiently increase to match NH_4^+ oxidation.

A key question, then, is why NO_2^- accumulation enhances O isotope exchange with water during soil nitrification. Under our experimental conditions, soil pH remained near neutral (7.2) due to TES buffering. Using pH 7.2 and the temperature–pH relationship of Buchwald and Casciotti (2013), estimated abiotic exchange rate constants at 28 and 21°C were 0.33 and 0.09 d^{-1} , corresponding to exchange half-lives of approximately 2 and 8 days, respectively. At these temperatures, appreciable NO_2^- accumulation persisted for only 4–6 days, and peak NO_2^- concentrations represented approximately 16% of cumulative NO_3^- production at the highest NH_4^+ amendment level (Fig. 4g,h). Even under the conservative assumption that this entire peak NO_2^- pool underwent complete equilibration with water, the maximum fraction of the cumulative NO_3^- pool that could have been affected by exchange would be only 0.16, substantially lower than the observed f_{ex} values of 0.48 and 0.63 at 28 and 21°C , respectively (Fig. 6b,c). The same conclusion applies at 14 and 7°C , where abiotic exchange half-lives increased to approximately 22 and 26 days, respectively, while peak NO_2^- concentrations remained below 11% of the corresponding cumulative NO_3^- production. These simple estimates indicate that abiotic exchange between the bulk NO_2^- pool and soil water cannot account for either the magnitude or the variability of the observed f_{ex} values.

Biologically mediated NO_2^- –water O isotope exchange during NH_4^+ oxidation may therefore provide the mechanistic basis for the observed association between NO_2^- accumulation and f_{ex} . It has been shown that AOB maintain substantially lower pH (e.g., >2



pH units lower; Weidinger et al., 2007) in their periplasmic spaces than in the surrounding environment, potentially through proton translocation associated with energy conservation (Schmidt et al., 2004; Keerio et al., 2020). Such localized acidity could increase the concentration of protonated nitrous acid (HNO_2) and thereby enhance NO_2^- –water O exchange within or near the cell envelope when extracellular NO_2^- availability is high. Another possibility is that O isotope exchange occurs through reversible enzymatic transformations involving NO_2^- and intermediates such as hydroxylamine (NH_2OH) and nitric oxide (NO). Recent studies have identified NO as a central intermediate in NO_2^- production pathways of both AOB and AOA. In AOB, NO is produced from NH_2OH and subsequently oxidized to NO_2^- by an as-yet unidentified enzyme (Caranto and Lancaster, 2017), whereas in AOA, NO can be generated from NO_2^- through nitrite reductase (NirK) activity and subsequently react with NH_2OH to regenerate NO_2^- (Kozłowski et al., 2016). Reversible NO_2^- – NO cycling could therefore provide an intracellular pathway for repeated O atom exchange, particularly if reversibility increases under conditions of elevated NO_2^- availability (Buchwald and Wankel, 2022; Martocello and Wankel, 2024).

In our case, the optimized model predicted daily $f_{\text{ex,AO}}$ values of 0.13–0.76, accounting for approximately three-quarters of total modeled O isotope exchange (Fig. S8). Using a dense enrichment culture of *Nitrosomonas europaea* ($\sim 10^{10}$ – 10^{11} cells mL^{-1}) supplied with high NH_4^+ (50 mM) and excess endogenous NO_2^- (50–150 mM), Andersson et al. (1982) observed rapid O incorporation into both newly produced and pre-existing NO_2^- , with exchanged NO_2^- exceeding the amount of NH_4^+ oxidized by up to threefold. In contrast, Casciotti et al. (2010) reported substantially lower exchange ($f_{\text{ex,AO}} = 0.10$ – 0.25) using dilute cultures ($\sim 10^6$ cells mL^{-1}) of four AOB species supplied with much lower NH_4^+ concentrations (5–50 μM). Although microbial abundance was not measured in this study, reported AOB abundances in agricultural soils are typically on the order of 10^6 to 10^8 cells g^{-1} soil (Hermansson and Lindgren, 2001; Okano et al., 2004), corresponding to approximately $\sim 10^5$ – 10^7 cells mL^{-1} under our slurry incubation conditions. This estimated range is broadly comparable to the cell densities used by Casciotti et al. (2010) but substantially lower than those in the enrichment culture of Andersson et al. (1982). Initial NH_4^+ concentrations in our amended treatments (0.25–1 mM) were likewise intermediate between those used in the two previous studies. Collectively, these comparisons support the plausibility of model-derived $f_{\text{ex,AO}}$ values and further suggest that biologically mediated NO_2^- –water O isotope exchange may be strongly influenced by soil-specific conditions regulating nitrifier abundance, physiological activity, and substrate availability.

6.2 Temperature Dependence of Kinetic Isotope Effects

Following the consumption of accumulated NO_2^- and the restoration of low NO_2^- concentrations, measured $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ gradually stabilized across all treatments and ultimately converged toward the $\delta^{18}\text{O}$ of incubation water in the unlabeled treatment ($\delta^{18}\text{O}_{\text{H}_2\text{O}} = -6.9\text{‰}$) (Fig. 5f–5j). Similarly, in scenario B1, modeled variability in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ decreased over time, reaching within $\pm 1.5\text{‰}$ of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ by 90 days after NH_4^+ amendment across all temperatures (Fig. 9a–9e). During this later stage, when NO_2^- accumulation was minimal, $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ is expected to be governed primarily by kinetic isotope effects associated with O atom incorporation. The limited variation in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ therefore suggests that these kinetic isotope effects are only weakly sensitive to temperature and to temperature- or substrate-driven changes in nitrification rates.

Consistent with this interpretation, the model-derived kinetic isotope effects ($^{18}\epsilon_{\text{k,comp}}$, $^{18}\epsilon_{\text{k,H}_2\text{O},2}$, and $^{18}\epsilon_{\text{k,NO}}$) exhibited only weak temperature dependence between 7 and 35 °C (Fig. 8). Theoretically, increasing temperature is expected to reduce the magnitude of equilibrium isotope effects by decreasing the difference in vibrational energies between isotopologues (Urey, 1947). Similar quantum mechanical and statistical approaches have also been used to approximate kinetic isotope effects, and for O isotopes, equilibrium and kinetic isotope effects are generally expected to be of comparable magnitude (Bigeleisen and Wolfsberg, 1958). Consistent with these predictions, Buchwald and Casciotti (2013) reported a linear decrease in the equilibrium isotope effect



between NO_2^- and water ($^{18}\epsilon_{\text{eq}}$) over 4–33 °C, with a temperature coefficient of approximately $-0.12\text{‰ }^\circ\text{C}^{-1}$ (Fig. 8). This value is comparable in magnitude to the modeled temperature coefficients for $^{18}\epsilon_{\text{k,comp}}$, $^{18}\epsilon_{\text{k,H}_2\text{O},2}$, and $^{18}\epsilon_{\text{k,NO}}$ (0.19, -0.09 , and $0.15\text{‰ }^\circ\text{C}^{-1}$, respectively; Table S3), lending qualitative support to the modeled estimates.

Interestingly, unlike $^{18}\epsilon_{\text{k,H}_2\text{O},2}$ and $^{18}\epsilon_{\text{k,NO}}$, the O isotope model predicted that $^{18}\epsilon_{\text{k,comp}}$ increased with temperature (Fig. 8). Similar temperature-dependent increases in isotope effects have been reported for microbial respiration and other carbon and sulfur transformations (Farquhar et al., 1989; Canfield et al., 2006; Stolper et al., 2018). Such patterns are generally attributed not to changes in the intrinsic isotope effect, but to shifts in the relative contributions of multiple reaction steps as temperature alters reaction or growth rates. For example, Stolper et al. (2018) showed that the apparent O isotope effect of aerobic respiration in *Escherichia coli* increased with temperature as isotopic control shifted from reversible O_2 binding to terminal oxidases under slow growth to irreversible O_2 reduction under faster growth. In our incubations, neither O_2 nor water was limiting, making diffusion-related isotope effects unlikely to explain the modeled temperature dependence. Thus, because NH_3 oxidation to NH_2OH by ammonia monooxygenase (AMO) is considered the rate-limiting step of NH_4^+ oxidation, temperature-dependent shifts in the relative contributions of reversible and irreversible O_2 activation steps during AMO catalysis may explain the modeled increase in $^{18}\epsilon_{\text{k,comp}}$.

6.3 Predictability of $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ under Variable Temperature and Substrate Availability

Results from scenario B2 showed that $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ varied linearly with $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ across an environmentally relevant range of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values (Fig. 10). However, at a given $\delta^{18}\text{O}_{\text{H}_2\text{O}}$, $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ varied by 3.4–5.3‰ in response to variations in temperature and substrate availability (Fig. 10). Given the weak temperature dependence of the kinetic isotope effects, this variability was driven primarily by variation in f_{ex} and the associated expression of the equilibrium isotope effect, which were, in turn, regulated by temperature and substrate availability through their effects on transient NO_2^- accumulation (see above). Specifically, higher substrate availability and low-to-intermediate temperatures ($<30^\circ\text{C}$) promoted greater NO_2^- accumulation, leading to more extensive O isotope exchange and consequently higher $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ values. In contrast, high temperatures ($>30^\circ\text{C}$) and low substrate availability limited NO_2^- accumulation and O isotope exchange, favoring lower $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ values that more directly reflected the kinetic isotope effects.

Overall, the magnitude of this modeled variability is broadly consistent with the synthesis of Boshier et al. (2019), which predicted that, at $\delta^{18}\text{O}_{\text{H}_2\text{O}} = -5\text{‰}$, $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ would generally fall within approximately $\pm 3\text{‰}$ of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. However, whereas Boshier et al. (2019) used Monte Carlo simulations based on the ranges of f_{ex} and kinetic isotope effects reported across multiple laboratory studies (Table 1), our results demonstrate that a comparable range of variability can arise from nitrification within a single soil under variable temperature and substrate availability. To our knowledge, this environmentally induced variability cannot currently be predicted from isotope theory alone and therefore must be constrained experimentally.

From an empirical perspective, the modeled linear relationships between $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ were broadly consistent with a 1:1 relationship (Fig. 10), supporting the use of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ as a predictor or normalization reference for $\delta^{18}\text{O}_{\text{NO}_3}$ under hydrologically static conditions. However, our simulations further indicate that deviations of $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ from source water $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ need not be symmetric. When NO_2^- –water O isotope exchange is incomplete (i.e., $f_{\text{ex}} < 1$), a fraction of the O atoms ultimately incorporated into NO_3^- is derived from O_2 . Assuming that the kinetic isotope effect associated with incorporation of this O_2 -derived O ($^{18}\epsilon_{\text{k,O}_2}$) is approximately one-half that of $^{18}\epsilon_{\text{k,comp}}$ (Granger and Wankel, 2016), the effective $\delta^{18}\text{O}$ of this O_2 -derived O is approximately 7‰. Consequently, the relative contribution of O_2 -derived O becomes increasingly important as $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ diverges from this value. Graphically, because the slope of the $\delta^{18}\text{O}_{\text{H}_2\text{O}}-\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ relationship is less than unity when $f_{\text{ex}} < 1$, $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ can be either higher or lower than $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ depending on the isotopic composition of the source water (Fig. 10). This mechanism explains why $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$



was consistently lower than $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in the ^{18}O -enriched water treatments and why $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in these treatments was particularly sensitive to variation in f_{ex} (Fig. 5). Conversely, when source water is strongly ^{18}O -depleted, as may occur in winter and spring precipitation in northern temperate regions (e.g., $\delta^{18}\text{O}_{\text{H}_2\text{O}} < -20\text{‰}$; [Bowen and Revenaugh, 2003](#)), nitrification-derived NO_3^- can be expected to be systematically enriched in ^{18}O relative to its source water.

6.4 Evaporative and Hydrological Controls on $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$

Results from the synthetic transport modeling in scenario HB1 demonstrate that evaporation can substantially modify both $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in the topsoil (Fig. 11). Simulated evaporative enrichment of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in the topsoil reached 3.2‰ when averaged over the simulation period and up to 8‰ at the daily scale (Fig. 11a,b). This magnitude of enrichment is consistent with field observations showing that topsoil water can be enriched by up to approximately 10‰ relative to deeper soil water across a range of climates and soil types (e.g., [Hsieh et al., 1998](#); [Hahm et al., 2020](#); [Tetzlaff et al., 2021](#)). Evaporation also substantially enriched $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in the topsoil, with enrichment reaching up to 4‰ at the daily scale and approximately 2‰ when integrated across the simulation period (Fig. 11e,f). Thus, evaporative effects on $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ are comparable in magnitude to those arising from biogeochemical controls over both short (≤ 14 days) and longer (90 days) timescales (Fig. 9a–e).

Importantly, the expression of evaporative enrichment in both $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ depended strongly on the age of water selected by evaporation (Fig. 11b,f). This strong age dependence highlights the role of pore-scale connectivity and flow path organization in regulating isotopic signatures through their control of water age selection. In topsoil storage, water is continuously depleted through evaporation, transpiration, and percolation and replenished by precipitation, such that recently infiltrated water can constitute a substantial fraction of total storage ([Benettin et al., 2022](#)). In contrast, individual older water generally contributes less to bulk storage because water that remains in the system longer has more opportunities to leave through one of the available outflow pathways ([Benettin et al., 2022](#)). In fine-textured topsoil, this older water fraction may be retained in smaller pores with lower hydraulic conductivity but can remain hydraulically connected to the soil surface through liquid-phase continuity and capillary forces ([Or et al., 2013](#)). Preferential removal of older water by evaporation would therefore further reduce the contribution of this older fraction to total storage, suppressing the evaporative enrichment expressed in bulk $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. In the limiting case where evaporation sequentially removes the oldest water from storage, little or no evaporative enrichment would develop in the remaining storage because the water most strongly fractionated by evaporation is continually removed. By contrast, preferential evaporation of younger water exposes a larger fraction of stored water to evaporative fractionation before it is exported through other pathways ([Sprenger et al., 2019](#)). This greater exposure therefore produces stronger enrichment in bulk $\delta^{18}\text{O}_{\text{H}_2\text{O}}$, which is subsequently propagated into $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$.

Unlike topsoil storage, $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and its evaporative enrichment in column discharge were additionally influenced by the age selection of percolation (Fig. 11d). Percolation provides the direct hydrologic connection between topsoil storage and the subsoil and, together with evaporation and transpiration, determines how water is partitioned along the soil profile. Preferential percolation of older water can therefore directly transfer the evaporatively enriched fraction of topsoil storage to discharge, whereas preferential percolation of younger water removes water before it undergoes substantial evaporative fractionation, thereby reducing transmission of the evaporative signal to subsoil storage and ultimately to discharge. In contrast, $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in column discharge remained primarily controlled by the age preference of evaporation and showed little sensitivity to k_R (Fig. 11h). This difference arises because nitrification was assumed to occur only in the topsoil. As a result, $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ records the isotopic composition of water during NO_3^- production in the topsoil and is subsequently transported without further isotopic modification during percolation.



The contrasting effects of percolation on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ provide a mechanistic explanation for the prediction biases in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$. When percolation preferentially exported younger water ($k_R < 1$), column discharge $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ was dominated by recently introduced water that had experienced relatively little evaporative enrichment. These young water parcels also contained relatively little NO_3^- because of their short residence times in the topsoil. Consequently, the exported NO_3^- pool and its associated $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ were weighted more strongly toward intermediate- and older-age water that had experienced greater cumulative evaporative enrichment. Using column discharge $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ to predict $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ therefore underestimated the evaporative enrichment recorded by NO_3^- , resulting in a negative prediction bias (Fig. 12).

Conversely, when percolation preferentially exported older water ($k_R > 1$), $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in subsoil storage and column discharge was more strongly enriched because the exported water had experienced greater cumulative evaporation. However, $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ associated with this older water does not simply reflect $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ at the time of export. Rather, it integrates the temporal evolution of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ during the period when water resided in the topsoil and accumulated NO_3^- . Thus, $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ retains a temporally integrated evaporative signal that reflects the isotopic history of water during its residence in the nitrification zone. When older water was preferentially exported, the instantaneous $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ of column discharge therefore overstated the evaporative enrichment represented by the associated $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$, resulting in a positive prediction bias (Fig. 12).

Recent $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ measurements across a range of spatial scales have shown stronger evaporative enrichment in soil water and plant transpiration than in mobile water draining the soil profile or contributing to streamflow (Brooks et al., 2010; Good et al., 2015; Evaristo et al., 2015; Sprenger et al., 2018). These observations have contributed to the ecohydrological separation or “two water worlds” hypothesis, which proposes that groundwater recharge and streamflow are dominated by mobile water transported through preferential flow pathways, whereas evapotranspiration is supported primarily by slower-moving and more strongly retained water in the soil matrix and capillary pores (McDonnell, 2014). Within our SAS-based transport framework, strong young water preference for percolation (e.g., $k_R < 0.5$) combined with strong old water preference for evaporation (e.g., $k_E > 5$) represents one possible expression of this separation (Knighton et al., 2019; Sprenger et al., 2022). Under such conditions, our synthetic transport modeling predicts relatively weak (i.e., $< \sim 1\text{‰}$) evaporative enrichment of both discharge $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ when integrated across the simulation period (Fig. 11). Nevertheless, negative prediction biases in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ can persist (Fig. 12), even when the $\delta^{18}\text{O}_{\text{H}_2\text{O}} - \delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ relationship during nitrification is precisely known. These results demonstrate that the evaporative enrichment expressed in soil drainage and streamflow depends strongly on the age composition and connectivity of water contributing to evaporation versus percolation. Moreover, because the water pools and flow paths primarily contributing to drainage and NO_3^- mass load can diverge during percolation, the evaporative signal recorded by $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in drainage need not be expressed to the same extent in the corresponding $\delta^{18}\text{O}_{\text{H}_2\text{O}}$.

6.5 Interacting Biogeochemical and Hydrological Controls and Implications for Estimating $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in Field Applications

When biogeochemical controls on nitrification, evaporative and transport processes, and the seasonal timing of nitrification were considered simultaneously (scenario HB2), the range of $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in column discharge expanded to approximately -9 to 1‰ (Fig. 13a). This range was approximately twice as wide as that predicted from biogeochemical controls alone, demonstrating that hydrological processes can substantially amplify $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ variability through their interactions with biogeochemical controls. Part of this expanded variability resulted from systematic prediction biases that emerged when nitrification was restricted to individual seasons. Specifically, spring- and summer-dominated nitrification produced systematic negative biases of up to -2.2‰ , whereas winter-only nitrification produced a systematic positive bias (Fig. 13b–f).

These seasonal biases can be explained by the seasonal partitioning of water between evaporation and percolation and the resulting differences in $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ along the soil profile. During spring and early summer, enhanced evaporation combined with isotopically



enriched precipitation inputs increased $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in topsoil storage (Fig. 11a), and this enriched water was subsequently incorporated into NO_3^- during nitrification. In contrast, $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in the subsoil and column discharge integrated percolation over longer periods and was influenced more strongly by precipitation during the non-growing season, when most recharge events occurred (Fig. 3c). Consequently, when nitrification occurred primarily during spring or summer, NO_3^- acquired a more ^{18}O -enriched signal than would be inferred from the contemporaneous $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ of column discharge. The pattern was reversed during winter, when lower $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in winter topsoil storage resulted in more ^{18}O -depleted $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ than would be predicted from column discharge $\delta^{18}\text{O}_{\text{H}_2\text{O}}$, which also reflected recharge occurring during other seasons (Fig. 3c).

These coupled simulations therefore demonstrate a fundamental spatial and temporal decoupling between the water represented by drainage $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and the water involved in NO_3^- production. Whereas $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in drainage systems integrates water of different ages transported along different flow paths, $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ can preferentially reflect the isotopic composition of water within nitrification zones at specific locations and times. This decoupling may be particularly important in lowland agricultural watersheds, such as the glaciated UER region, where large groundwater stores can substantially dampen seasonal variability in streamflow $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ (Miller et al., 2026), while streamflow NO_3^- concentrations and isotopic compositions remain highly responsive to seasonal fertilizer inputs (Sha et al., 2025). Consequently, streamflow $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ measurements may provide a biased representation of the water from which nitrification-derived NO_3^- acquired its isotopic composition.

These findings highlight the need to account for water age, flow path organization, and the timing of nitrification relative to evaporation and percolation when applying $\delta^{18}\text{O}_{\text{H}_2\text{O}} - \delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ relationships at watershed scales. More broadly, NO_3^- isotope applications would benefit from moving beyond a stream-centric perspective toward a reactive transport framework that explicitly considers the diversity of flow paths and water ages within watersheds and their interactions with N biogeochemistry (Li et al., 2021). To that end, coupling NO_3^- isotope measurements with conceptual hydrological transport approaches, such as SAS functions, and process-based N biogeochemical models could provide a model-based framework for resolving these interactions and improving the prediction of $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ at the watershed scale.

6.6 Assumptions and Limitations

Several assumptions and limitations should be considered when interpreting the results presented here. First, the incubation experiments were conducted using a single agricultural soil, and different soils may harbor distinct nitrifying communities and exhibit different responses to temperature and substrate availability. The extent to which the results can be generalized across soil types therefore requires further investigation.

Second, coupling the slurry incubation results with HYDRUS-1D and SAS-based transport simulations necessarily simplifies the pore-scale heterogeneity governing evaporation, percolation, and microbial activity. For example, the SAS parameterizations used to represent evaporation and percolation are time-invariant and therefore do not account for temporal changes in flow paths contributing to these processes. In natural systems, different flow paths may become active under changing hydrological conditions, resulting in greater temporal variability in water velocities and ages (Harman, 2015). Similarly, our simulations assumed that nitrification occurs uniformly throughout the topsoil, representing a “well-mixed” biogeochemical scenario. In reality, microbial activity may be concentrated within specific microsites or along particular flow paths because of spatial heterogeneity in soil physical and chemical properties that regulate redox conditions and nutrient availability at the pore scale (Franklin et al., 2019). Such heterogeneity could therefore associate nitrification disproportionately with particular locations and water ages within the topsoil, leading to departures from the assumed biogeochemical mixing. Despite these simplifications, the modeling framework provides a controlled means of isolating biogeochemical and hydrological controls on $\delta^{18}\text{O}_{\text{NO}_3, \text{nit}}$ that would be difficult, if not impossible, to separate under field conditions. Moreover, incorporating temporal and spatial heterogeneity would



likely increase, rather than decrease, the variability in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$, particularly over short timescales. The variability quantified in this study should therefore be considered a conservative estimate of the variability that may occur under natural conditions.

820 Finally, our simulations do not explicitly represent NO_3^- sinks such as denitrification and microbial assimilation. Although these processes do not directly modify the $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ acquired during nitrification, selective removal of NO_3^- can alter the $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ of the remaining NO_3^- pool and therefore change the isotopic weighting of NO_3^- reaching drainage systems and streams. Incorporating dynamic SAS functions, spatially explicit or water age-specific nitrification, and downstream NO_3^- transformations within a fully coupled reactive transport framework will be important for evaluating these effects and for testing the mechanisms identified here

825 against field observations.

7 Conclusion

In this study, we combined soil incubation experiments with a mechanistic biogeochemical model to quantify how temperature, NH_4^+ availability, and soil water $\delta^{18}\text{O}$ regulate $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ in an agricultural soil. We further coupled biogeochemical $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ simulations with a water age-based synthetic transport model to evaluate how soil evaporation influences the variability and predictability of $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ under different water transport regimes. Together, this coupled framework provides a mechanistic basis for evaluating $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ across a diverse range of interacting biogeochemical and hydrological conditions.

The incubation experiments showed that, across wide ranges of temperature (7–35 °C) and NH_4^+ availability (0–98 mg N kg⁻¹ soil), $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ generally tracked soil water $\delta^{18}\text{O}$ but varied by 3.4–5.3‰ at a given $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ during active nitrification. Temperature and substrate availability influenced $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ primarily through their effects on transient NO_2^- accumulation, which enhanced

835 NO_2^- –water O isotope exchange and expression of the associated equilibrium isotope effect. Higher substrate availability and low-to-intermediate temperatures (<30 °C) promoted greater NO_2^- accumulation, resulting in more extensive O isotope exchange and higher $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ values. In contrast, high temperatures (>30 °C) and low substrate availability limited NO_2^- accumulation and O isotope exchange, favoring lower $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ values, which are more reflective of the kinetic isotope effects associated with nitrification.

840 The synthetic transport modeling further demonstrated that soil evaporation can increase $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ variability in nitrification source zones and soil drainage to a magnitude comparable to that associated with the biogeochemical controls. The expression of evaporative enrichment in $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ is primarily determined by the sources and ages of water participating in evaporation, whereas drainage $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ is additionally shaped by the sources, ages, and transport pathways of water contributing to percolation. As a result, $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in drainage can retain different evaporative signals, producing systematic biases when drainage

845 $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ is used to predict $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$. These biases are particularly pronounced when the timing of nitrification is decoupled from percolation and when the water contributing to drainage differs in age from the water involved in NO_3^- production.

Overall, these findings demonstrate that $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ is controlled by interacting biogeochemical and hydrological processes operating across different spatial and temporal scales. These processes can both modify the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ – $\delta^{18}\text{O}_{\text{NO}_3,\text{nit}}$ relationship during soil nitrification and decouple the isotopic composition of nitrification-derived NO_3^- from that of water sampled in drainage or

850 streamflow. Future applications of NO_3^- isotope tracers should therefore move beyond a stream-centric perspective and adopt a reactive transport framework that explicitly accounts for dynamic biogeochemical controls on nitrification across diverse flow paths and water ages within soils and watersheds.



Code and data availability

The incubation data are available at <https://doi.org/10.5281/zenodo.22684050>. The dynamic nitrification model is available at
855 <https://doi.org/10.5281/zenodo.22740286>. The transport modeling was built upon the open-source trans-SAS v1.0 package
(Benettin & Bertuzzo, 2018a), available at <https://doi.org/10.5281/zenodo.1203600>.

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Author contributions

860 MS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original
draft. ZY: Conceptualization, Data curation, Funding acquisition, Methodology, Supervision, Project administration, Resources,
Validation, Writing – review & editing.

Competing interests

The authors declare that they have no conflict of interest.

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References

- 870 Andersson, K. K., Philson, S. B., and Hooper, A. B.: ^{18}O isotope shift in ^{15}N NMR analysis of biological N-oxidations: H_2O - NO_2^- exchange in the ammonia-oxidizing bacterium *Nitrosomonas*, Proc. Natl. Acad. Sci. USA, 79, 5871–5875, <https://doi.org/10.1073/pnas.79.19.5871>, 1982.
Angert, A., Barkan, E., Barnett, B., Brugnoli, E., Davidson, E. A., Fessenden, J., Manepong, S., Panapitukkul, N., Randerson, J. T., Savage, K., Yakir, D., and Luz, B.: Contribution of soil respiration in tropical, temperate, and boreal forests to the ^{18}O enrichment of atmospheric O_2 , Glob. Biogeochem. Cycles, 17, <https://doi.org/10.1029/2003gb002056>, 2003.
- 875 Asadollahi, M., Stumpp, C., Rinaldo, A., and Benettin, P.: Transport and water age dynamics in soils: A comparative study of spatially integrated and spatially explicit models, Water Resour. Res., 56, e2019WR025539, <https://doi.org/10.1029/2019WR025539>, 2020.
Benettin, P. and Bertuzzo, E.: tran-SAS v1.0, Zenodo [code], <https://doi.org/10.5281/zenodo.1203600>, 2018a.
- 880 Benettin, P. and Bertuzzo, E.: tran-SAS v1.0: A numerical model to compute catchment-scale hydrologic transport using StorAge Selection functions, Geosci. Model Dev., 11, 1627–1639, <https://doi.org/10.5194/gmd-11-1627-2018>, 2018b.
Benettin, P., van der Velde, Y., van der Zee, S. E. A. T. M., Rinaldo, A., and Botter, G.: Chloride circulation in a lowland catchment and the formulation of transport by travel time distributions, Water Resour. Res., 49, 4619–4632, <https://doi.org/10.1002/wrcr.20309>, 2013.
- 885 Benettin, P., Soulsby, C., Birkel, C., Tetzlaff, D., Botter, G., and Rinaldo, A.: Using SAS functions and high-resolution isotope data to unravel travel time distributions in headwater catchments, Water Resour. Res., 53, 1864–1878, <https://doi.org/10.1002/2016WR020117>, 2017.
Benettin, P., Rodriguez, N. B., Sprenger, M., Kim, M., Klaus, J., Harman, C. J., van der Velde, Y., Hrachowitz, M., Botter, G., McGuire, K. J., Kirchner, J. W., Rinaldo, A., and McDonnell, J. J.: Transit time estimation in catchments: Recent developments and future directions, Water Resour. Res., 58, e2022WR033096, <https://doi.org/10.1029/2022WR033096>, 2022.
- 890 Bigeleisen, J. and Wolfsberg, M.: Theoretical and experimental aspects of isotope effects in chemical kinetics, in: Advances in Chemical Physics, edited by: Prigogine, I. and Debye, P., 1st edn., Vol. 1, Wiley, 15–76, <https://doi.org/10.1002/9780470143476.ch2>, 1958.
Bonner, F. T.: Oxygen exchange between nitric oxide and water, Inorg. Chem., 9, 190–193, <https://doi.org/10.1021/ic50083a039>, 1970.
- 895 Boshers, D. S., Granger, J., Tobias, C. R., Bohlke, J. K., and Smith, R. L.: Constraining the oxygen isotopic composition of nitrate produced by nitrification, Environ. Sci. Technol., 53, 1206–1216, <https://doi.org/10.1021/acs.est.8b03386>, 2019.
Botter, G., Bertuzzo, E., and Rinaldo, A.: Catchment residence and travel time distributions: The master equation, Geophys. Res. Lett., 38, L11403, <https://doi.org/10.1029/2011GL047666>, 2011.
- 900 Bowen, G. J. and Revenaugh, J.: Interpolating the isotopic composition of modern meteoric precipitation, Water Resour. Res., 39, 1299, <https://doi.org/10.1029/2003WR002086>, 2003.
Brooks, J. R., Barnard, H. R., Coulombe, R., and McDonnell, J. J.: Ecohydrologic separation of water between trees and streams in a Mediterranean climate, Nat. Geosci., 3, 100–104, <https://doi.org/10.1038/NCEO722>, 2010.
Buchwald, C. and Casciotti, K. L.: Oxygen isotopic fractionation and exchange during bacterial nitrite oxidation, Limnol. Oceanogr., 55, 1064–1074, <https://doi.org/10.4319/lo.2010.55.3.1064>, 2010.
- 905 Buchwald, C. and Casciotti, K. L.: Isotopic ratios of nitrite as tracers of the sources and age of oceanic nitrite, Nat. Geosci., 6, 308–313, <https://doi.org/10.1038/ngeo1745>, 2013.



- Buchwald, C. and Wankel, S. D.: Enzyme-catalyzed isotope equilibrium: A hypothesis to explain apparent N cycling phenomena in low oxygen environments, *Mar. Chem.*, 244, 104140, <https://doi.org/10.1016/j.marchem.2022.104140>, 2022.
- 910 Buchwald, C., Santoro, A. E., McIlvin, M. R., and Casciotti, K. L.: Oxygen isotopic composition of nitrate and nitrite produced by nitrifying cocultures and natural marine assemblages, *Limnol. Oceanogr.*, 57, 1361–1375, <https://doi.org/10.4319/lo.2012.57.5.1361>, 2012.
- Canfield, D. E., Olesen, C. A., and Cox, R. P.: Temperature and its control of isotope fractionation by a sulfate-reducing bacterium, *Geochim. Cosmochim. Acta*, 70, 548–561, <https://doi.org/10.1016/j.gca.2005.10.028>, 2006.
- 915 Caranto, J. D. and Lancaster, K. M.: Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase, *Proc. Natl. Acad. Sci. USA*, 114, 8217–8222, <https://doi.org/10.1073/pnas.1704504114>, 2017.
- Casciotti, K. L., Sigman, D. M., Hastings, M. G., Bohlke, J. K., and Hilkert, A.: Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method, *Anal. Chem.*, 74, 4905–4912, <https://doi.org/10.1021/ac020113w>, 2002.
- 920 Casciotti, K. L., Bohlke, J. K., McIlvin, M. R., Mroczkowski, S. J., and Hannon, J. E.: Oxygen isotopes in nitrite: Analysis, calibration, and equilibration, *Anal. Chem.*, 79, 2427–2436, <https://doi.org/10.1021/ac061598h>, 2007.
- Casciotti, K. L., McIlvin, M., and Buchwald, C.: Oxygen isotopic exchange and fractionation during bacterial ammonia oxidation, *Limnol. Oceanogr.*, 55, 753–762, <https://doi.org/10.4319/lo.2010.55.2.0753>, 2010.
- Craig, H. and Gordon, L. I.: Deuterium and oxygen-18 variations in the ocean and the marine atmosphere, in: *Stable Isotopes in Oceanographic Studies and Paleotemperatures*, edited by: Tongiorgi, E., Consiglio nazionale delle ricerche, Laboratorio di geologia nucleare, Pisa, Italy, 9–130, 1965.
- 925 DiSpirito, A. A. and Hooper, A. B.: Oxygen exchange between nitrate molecules during nitrite oxidation by *Nitrobacter*, *J. Biol. Chem.*, 261, 10534–10537, [https://doi.org/10.1016/S0021-9258\(18\)67417-4](https://doi.org/10.1016/S0021-9258(18)67417-4), 1986.
- Doherty, J.: PEST model-independent parameter estimation, 5th edn., Watermark Numerical Computing, Brisbane, Australia, 2004.
- 930 Duan, P., Zhang, Q., and Xiong, Z.: Temperature decouples ammonia and nitrite oxidation in greenhouse vegetable soils, *Sci. Total Environ.*, 733, 139391, <https://doi.org/10.1016/j.scitotenv.2020.139391>, 2020.
- Evaristo, J., Jasechko, S., and McDonnell, J. J.: Global separation of plant transpiration from groundwater and streamflow, *Nature*, 525, 91–94, <https://doi.org/10.1038/nature14983>, 2015.
- 935 Farquhar, G. D., Hubick, K. T., Condon, A. G., and Richards, R. A.: Carbon isotope fractionation and plant water-use efficiency, in: *Stable Isotopes in Ecological Research*, edited by: Rundel, P. W., Ehleringer, J. R., and Nagy, K. A., Vol. 68, Springer New York, 21–40, https://doi.org/10.1007/978-1-4612-3498-2_2, 1989.
- Franklin, S., Vasilas, B., and Jin, Y.: More than meets the dye: Evaluating preferential flow paths as microbial hotspots, *Vadose Zone J.*, 18, 1–8, <https://doi.org/10.2136/vzj2019.03.0024>, 2019.
- 940 Giguere, A. T., Taylor, A. E., Myrold, D. D., Mellbye, B. L., Sayavedra-Soto, L. A., and Bottomley, P. J.: Nitrite-oxidizing activity responds to nitrite accumulation in soil, *FEMS Microbiol. Ecol.*, 94, fyy008, <https://doi.org/10.1093/femsec/fyy008>, 2018.
- Good, S. P., Noone, D., and Bowen, G.: Hydrologic connectivity constrains partitioning of global terrestrial water fluxes, *Science*, 349, 175–177, <https://doi.org/10.1126/science.aaa5931>, 2015.
- Granger, J. and Sigman, D. M.: Removal of nitrite with sulfamic acid for nitrate N and O isotope analysis with the denitrifier method, *Rapid Commun. Mass Spectrom.*, 23, 3753–3762, <https://doi.org/10.1002/rcm.4307>, 2009.
- 945 Granger, J. and Wankel, S. D.: Isotopic overprinting of nitrification on denitrification as a ubiquitous and unifying feature of environmental nitrogen cycling, *Proc. Natl. Acad. Sci. USA*, 113, E6391–E6400, <https://doi.org/10.1073/pnas.1601383113>, 2016.



- Hahm, W. J., Rempe, D. M., Dralle, D. N., Dawson, T. E., and Dietrich, W. E.: Oak transpiration drawn from the weathered bedrock vadose zone in the summer dry season, *Water Resour. Res.*, 56, e2020WR027419, <https://doi.org/10.1029/2020WR027419>, 2020.
- Harman, C. J.: Time-variable transit time distributions and transport: theory and application to storage-dependent transport of chloride in a watershed, *Water Resour. Res.*, 51, 1–30, <https://doi.org/10.1002/2014WR015707>, 2015.
- Hermansson, A. and Lindgren, P.-E.: Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR, *Appl. Environ. Microbiol.*, 67, 972–976, <https://doi.org/10.1128/AEM.67.2.972-976.2001>, 2001.
- Hollocher, T. C.: Source of the oxygen atoms of nitrate in the oxidation of nitrite by *Nitrobacter agilis* and evidence against a P-O-N anhydride mechanism in oxidative phosphorylation, *Arch. Biochem. Biophys.*, 233, 721–727, [https://doi.org/10.1016/0003-9861\(84\)90499-5](https://doi.org/10.1016/0003-9861(84)90499-5), 1984.
- Hsieh, J. C. C., Chadwick, O. A., Kelly, E. F., and Savin, S. M.: Oxygen isotopic composition of soil water: Quantifying evaporation and transpiration, *Geoderma*, 82, 269–293, [https://doi.org/10.1016/S0016-7061\(97\)00105-5](https://doi.org/10.1016/S0016-7061(97)00105-5), 1998.
- Hu, Y., Yu, Z., Yang, W. H., Margenot, A. J., Gentry, L. E., Wander, M. M., Mulvaney, R. L., Mitchell, C. A., and Guacho, C. E.: Deciphering the isotopic imprint of nitrate to reveal nitrogen source and transport mechanisms in a tile-drained agroecosystem, *J. Geophys. Res. Biogeosciences*, 129, e2024JG008027, <https://doi.org/10.1029/2024JG008027>, 2024.
- Jones, R. W. and Hedlin, R. A.: Ammonium, nitrite and nitrate accumulation in three Manitoba soils as influenced by added ammonium sulfate and urea, *Can. J. Soil Sci.*, 50, 331–338, <https://doi.org/10.4141/cjss70-043>, 1970.
- Keerio, H. A., Bae, W., Park, J., and Kim, M.: Substrate uptake, loss, and reserve in ammonia-oxidizing bacteria (AOB) under different substrate availabilities, *Process Biochem.*, 91, 303–310, <https://doi.org/10.1016/j.procbio.2019.12.023>, 2020.
- Kendall, C., Elliott, E. M., and Wankel, S. D.: Tracing anthropogenic inputs of nitrogen to ecosystems, in: *Stable Isotopes in Ecology and Environmental Science*, edited by: Michener, R. and Lajtha, K., Blackwell Publishing Ltd, Oxford, UK, 375–449, <https://doi.org/10.1002/9780470691854.ch12>, 2007.
- Kirchner, J. W., Tetzlaff, D., and Soulsby, C.: Comparing chloride and water isotopes as hydrological tracers in two Scottish catchments, *Hydrol. Process.*, 24, 1631–1645, <https://doi.org/10.1002/hyp.7676>, 2010.
- Knighton, J., Souter-Kline, V., Volkmann, T., Troch, P. A., Kim, M., Harman, C. J., Morris, C., Buchanan, B., and Walter, M. T.: Seasonal and topographic variations in ecohydrological separation within a small, temperate, snow-influenced catchment, *Water Resour. Res.*, 55, 6417–6435, <https://doi.org/10.1029/2019WR025174>, 2019.
- Kozłowski, J. A., Stieglmeier, M., Schleper, C., Klotz, M. G., and Stein, L. Y.: Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota, *ISME J.*, 10, 1836–1845, <https://doi.org/10.1038/ismej.2016.2>, 2016.
- Kuppel, S., Tetzlaff, D., Maneta, M. P., and Soulsby, C.: EcH2O-iso 1.0: Water isotopes and age tracking in a process-based, distributed ecohydrological model, *Geosci. Model Dev.*, 11, 3045–3069, <https://doi.org/10.5194/gmd-11-3045-2018>, 2018.
- Li, L., Sullivan, P. L., Benettin, P., Cirpka, O. A., Bishop, K., Brantley, S. L., Knapp, J. L. A., Meerveld, I., Rinaldo, A., Seibert, J., Wen, H., and Kirchner, J. W.: Toward catchment hydro-biogeochemical theories, *WIREs Water*, 8, e1495, <https://doi.org/10.1002/wat2.1495>, 2021.
- Maggi, F. and Riley, W. J.: Transient competitive complexation in biological kinetic isotope fractionation explains nonsteady isotopic effects: Theory and application to denitrification in soils, *J. Geophys. Res.*, 114, G04012, <https://doi.org/10.1029/2008JG000815>, 2009.
- Maggi, F. and Riley, W. J.: The effect of temperature on the rate, affinity, and ^{15}N fractionation of NO_3^- during biological denitrification in soils, *Biogeochemistry*, 124, 235–253, <https://doi.org/10.1007/s10533-015-0095-2>, 2015.



- Maggi, F., Tang, F. H. M., and Riley, W. J.: The thermodynamic links between substrate, enzyme, and microbial dynamics in Michaelis–Menten–Monod kinetics, *Int. J. Chem. Kinet.*, 50, 343–356, <https://doi.org/10.1002/kin.21163>, 2018.
- 990 Maharjan, B. and Venterea, R. T.: Nitrite intensity explains N management effects on N₂O emissions in maize, *Soil Biol. Biochem.*, 66, 229–238, <https://doi.org/10.1016/j.soilbio.2013.07.015>, 2013.
- Martocello, D. E. and Wankel, S. D.: Physiological influence of Fe and Cu availability on nitrogen isotope fractionation during ammonia oxidation, *Environ. Sci. Technol.*, 58, 421–431, <https://doi.org/10.1021/acs.est.3c05964>, 2024.
- Matiatos, I., Wassenaar, L. I., Monteiro, L. R., Venkiteswaran, J. J., Gooddy, D. C., Boeckx, P., Sacchi, E., Yue, F. J., Michalski, 995 G., Peek, S., Steingruber, S., Tosheva, Z., Vitvar, T., and Marx, A.: Global patterns of nitrate isotope composition in rivers and adjacent aquifers reveal reactive nitrogen cascading, *Commun. Earth Environ.*, 2, 52, <https://doi.org/10.1038/s43247-021-00121-x>, 2021.
- McDonnell, J. J.: The two water worlds hypothesis: Ecohydrological separation of water between streams and trees?, *WIREs Water*, 1, 323–329, <https://doi.org/10.1002/wat2.1027>, 2014.
- 1000 Miller, S. G., Yu, Z., Sha, M., Hu, M., Guacho, C. E., and Mitchell, C. A.: Variable source zones controlling flow, water age, and nitrate export dynamics in a tile-drained lowland watershed, *Water Resour. Res.*, 62, e2025WR043120, <https://doi.org/10.1029/2025WR043120>, 2026.
- Monod, J.: The growth of bacterial cultures, *Annu. Rev. Microbiol.*, 3, 371–394, <https://doi.org/10.1146/annurev.mi.03.100149.002103>, 1949.
- 1005 Ni, B.-J., Fang, F., Xie, W.-M., and Yu, H.-Q.: Growth, maintenance and product formation of autotrophs in activated sludge: taking the nitrite-oxidizing bacteria as an example, *Water Res.*, 42, 4261–4270, <https://doi.org/10.1016/j.watres.2008.06.024>, 2008.
- Nishizawa, M., Sakai, S., Konno, U., Nakahara, N., Takaki, Y., Saito, Y., Imachi, H., Tasumi, E., Makabe, A., Koba, K., and Takai, K.: Nitrogen and oxygen isotope effects of ammonia oxidation by thermophilic *Thaumarchaeota* from a geothermal water stream, *Appl. Environ. Microbiol.*, 82, 4492–4504, <https://doi.org/10.1128/AEM.00250-16>, 2016.
- 1010 NOAA: Historical daily conditions of Tuscola, IL (USC00118684), <https://www.nci.noaa.gov/maps-and-geospatial-products> (last access: 21 December 2025), 2022.
- Okano, Y., Hristova, K. R., Leutenegger, C. M., Jackson, L. E., Denison, R. F., Gebreyesus, B., Lebauer, D., and Scow, K. M.: Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil, *Appl. Environ. Microbiol.*, 70, 1008–1016, <https://doi.org/10.1128/AEM.70.2.1008-1016.2004>, 2004.
- 1015 Or, D., Lehmann, P., Shahraeeni, E., and Shokri, N.: Advances in soil evaporation physics – a review, *Vadose Zone J.*, 12, 1–16, <https://doi.org/10.2136/vzj2012.0163>, 2013.
- Ouyang, Y., Norton, J. M., and Stark, J. M.: Ammonium availability and temperature control contributions of ammonia oxidizing bacteria and archaea to nitrification in an agricultural soil, *Soil Biol. Biochem.*, 113, 161–172, <https://doi.org/10.1016/j.soilbio.2017.06.010>, 2017.
- 1020 Queloz, P., Bertuzzo, E., Carraro, L., Botter, G., Miglietta, F., Rao, P. S. C., and Rinaldo, A.: Transport of fluorobenzoate tracers in a vegetated hydrologic control volume: 1. Experimental results, *Water Resour. Res.*, 51, 2773–2792, <https://doi.org/10.1002/2014WR016433>, 2015.
- Schaap, M. G., Leij, F. J., and Van Genuchten, M. Th.: Rosetta: A computer program for estimating soil hydraulic parameters with hierarchical pedotransfer functions, *J. Hydrol.*, 251, 163–176, [https://doi.org/10.1016/s0022-1694\(01\)00466-8](https://doi.org/10.1016/s0022-1694(01)00466-8), 2001.
- 1025 Schaefer, S. C. and Hollibaugh, J. T.: Temperature decouples ammonium and nitrite oxidation in coastal waters, *Environ. Sci. Technol.*, 51, 3157–3164, <https://doi.org/10.1021/acs.est.6b03483>, 2017.



- Schmidt, I., Look, C., Bock, E., and Jetten, M. S. M.: Ammonium and hydroxylamine uptake and accumulation in *Nitrosomonas*, *Microbiology*, 150, 1405–1412, <https://doi.org/10.1099/mic.0.26719-0>, 2004.
- Schwemmler, R. and Weiler, M.: Consistent modeling of transport processes and travel times – coupling soil hydrologic processes with StorAge Selection functions, *Water Resour. Res.*, 60, e2023WR034441, <https://doi.org/10.1029/2023WR034441>, 2023.
- 1030 Sha, M. and Yu, Z.: Biogeochemical and hydrological controls on the oxygen isotopic composition of nitrification-produced nitrate in an agricultural soil [code]. Zenodo. <https://doi.org/10.5281/zenodo.22684050>, 2026.
- Sha, M. and Yu, Z.: Biogeochemical and hydrological controls on the oxygen isotopic composition of nitrification-produced nitrate in an agricultural soil [data set]. Zenodo. <https://doi.org/10.5281/zenodo.22740286>, 2026.
- 1035 Sha, M., Yu, Z., Benettin, P., Gentry, L. E., and Mitchell, C. A.: Coupled hydrologic and biogeochemical responses of nitrate export in a tile-drained agricultural watershed revealed by SAS functions and nitrate isotopes, *Water Resour. Res.*, 61, e2024WR039718, <https://doi.org/10.1029/2024WR039718>, 2025.
- Simunek, J., Van Genuchten, M. Th., and Sejna, M.: Recent developments and applications of the HYDRUS computer software packages, *Vadose Zone J.*, 15, 1–25, <https://doi.org/10.2136/vzj2016.04.0033>, 2016.
- 1040 Smith, R. V., Doyle, R. M., Burns, L. C., and Stevens, R. J.: A model for nitrite accumulation in soils, *Soil Biol. Biochem.*, 29, 1241–1247, [https://doi.org/10.1016/S0038-0717\(97\)00028-X](https://doi.org/10.1016/S0038-0717(97)00028-X), 1997.
- Snider, D. M., Spoelstra, J., Schiff, S. L., and Venkiteswaran, J. J.: Stable oxygen isotope ratios of nitrate produced from nitrification: ^{18}O -labeled water incubations of agricultural and temperate forest soils, *Environ. Sci. Technol.*, 44, 5358–5364, <https://doi.org/10.1021/es1002567>, 2010.
- 1045 Spoelstra, J., Schiff, S. L., Hazlett, P. W., Jeffries, D. S., and Semkin, R. G.: The isotopic composition of nitrate produced from nitrification in a hardwood forest floor, *Geochim. Cosmochim. Acta*, 71, 3757–3771, <https://doi.org/10.1016/j.gca.2007.05.021>, 2007.
- Sprenger, M., Leistert, H., Gimbel, K., and Weiler, M.: Illuminating hydrological processes at the soil-vegetation-atmosphere interface with water stable isotopes, *Rev. Geophys.*, 54, 674–704, <https://doi.org/10.1002/2015RG000515>, 2016.
- 1050 Sprenger, M., Tetzlaff, D., Buttle, J., Laudon, H., Leistert, H., Mitchell, C. P. J., Snelgrove, J., Weiler, M., and Soulsby, C.: Measuring and modeling stable isotopes of mobile and bulk soil water, *Vadose Zone J.*, 17, 170149, <https://doi.org/10.2136/vzj2017.08.0149>, 2018.
- Sprenger, M., Stumpp, C., Weiler, M., Aeschbach, W., Allen, S. T., Benettin, P., Dubbert, M., Hartmann, A., Hrachowitz, M., Kirchner, J. W., McDonnell, J. J., Orlowski, N., Penna, D., Pfahl, S., Rinderer, M., Rodriguez, N., Schmidt, M., and Werner, C.: The demographics of water: A review of water ages in the critical zone, *Rev. Geophys.*, 57, 800–834, <https://doi.org/10.1029/2018RG000633>, 2019.
- 1055 Sprenger, M., Llorens, P., Gallart, F., Benettin, P., Allen, S. T., and Latron, J.: Precipitation fate and transport in a Mediterranean catchment through models calibrated on plant and stream water isotope data, *Hydrol. Earth Syst. Sci.*, 26, 4093–4107, <https://doi.org/10.5194/hess-26-4093-2022>, 2022.
- 1060 Stolper, D. A., Fischer, W. W., and Bender, M. L.: Effects of temperature and carbon source on the isotopic fractionations associated with O_2 respiration for $^{17}\text{O}/^{16}\text{O}$ and $^{18}\text{O}/^{16}\text{O}$ ratios in *E. coli*, *Geochim. Cosmochim. Acta*, 240, 152–172, <https://doi.org/10.1016/j.gca.2018.07.039>, 2018.
- Tappe, W., Laverman, A., Bohland, M., Braster, M., Rittershaus, S., Groeneweg, J., and Van Verseveld, H. W.: Maintenance energy demand and starvation recovery dynamics of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* cultivated in a retentostat with complete biomass retention, *Appl. Environ. Microbiol.*, 65, 2471–2477, <https://doi.org/10.1128/AEM.65.6.2471-2477.1999>, 1999.
- 1065



- Taylor, A. E., Zeglin, L. H., Dooley, S., Myrold, D. D., and Bottomley, P. J.: Evidence for different contributions of archaea and bacteria to the ammonia-oxidizing potential of diverse Oregon soils, *Appl. Environ. Microbiol.*, 76, 7691–7698, <https://doi.org/10.1128/AEM.01324-10>, 2010.
- 1070 Taylor, A. E., Giguere, A. T., Zoebelin, C. M., Myrold, D. D., and Bottomley, P. J.: Modeling of soil nitrification responses to temperature reveals thermodynamic differences between ammonia-oxidizing activity of archaea and bacteria, *ISME J.*, 11, 896–908, <https://doi.org/10.1038/ismej.2016.179>, 2017.
- Taylor, A. E., Myrold, D. D., and Bottomley, P. J.: Temperature affects the kinetics of nitrite oxidation and nitrification coupling in four agricultural soils, *Soil Biol. Biochem.*, 136, 107523, <https://doi.org/10.1016/j.soilbio.2019.107523>, 2019.
- 1075 Tetzlaff, D., Buttle, J., Carey, S. K., Kohn, M. J., Laudon, H., McNamara, J. P., Smith, A., Sprenger, M., and Soulsby, C.: Stable isotopes of water reveal differences in plant – soil water relationships across northern environments, *Hydrol. Process.*, 35, e14023, <https://doi.org/10.1002/hyp.14023>, 2021.
- Turk, H., Stumpp, C., Hrachowitz, M., Strauss, P., Bloschl, G., and Stockinger, M.: Catchment transit time variability with different SAS function parameterizations for the unsaturated zone and groundwater, *Hydrol. Earth Syst. Sci.*, 30, 1053–1076, <https://doi.org/10.5194/hess-30-1053-2026>, 2026.
- 1080 Urey, H. C.: The thermodynamic properties of isotopic substances, *J. Chem. Soc. Resumed*, 562, <https://doi.org/10.1039/jr9470000562>, 1947.
- USDA-NRCS: Gridded soil survey geographic (gSSURGO) database for Champaign county, Illinois [data set], <https://gdg.sc.egov.usda.gov/> (last access: 2 July 2025), 2016.
- 1085 van der Velde, Y., Torfs, P. J. J. F., van der Zee, S. E. A. T. M., and Uijlenhoet, R.: Quantifying catchment-scale mixing and its effect on time-varying travel time distributions, *Water Resour. Res.*, 48, W06536, <https://doi.org/10.1029/2011WR011310>, 2012.
- Venkiteswaran, J. J., Boeckx, P., and Goddy, D. C.: Towards a global interpretation of dual nitrate isotopes in surface waters, *J. Hydrol. X*, 4, 100037, <https://doi.org/10.1016/j.hydroa.2019.100037>, 2019.
- Venterea, R. T., Coulter, J. A., and Clough, T. J.: Nitrite accumulation and nitrogen gas production increase with decreasing temperature in urea-amended soils: Experiments and modeling, *Soil Biol. Biochem.*, 142, 107727, <https://doi.org/10.1016/j.soilbio.2020.107727>, 2020.
- 1090 WARM: Illinois Climate Network, Illinois State Water Survey [data set], <https://doi.org/10.13012/J8MW2F2Q>, 2015.
- Weidinger, K., Neuhauser, B., Gilch, S., Ludewig, U., Meyer, O., and Schmidt, I.: Functional and physiological evidence for a Rhesus-type ammonia transporter in *Nitrosomonas europaea*, *FEMS Microbiol. Lett.*, 273, 260–267, <https://doi.org/10.1111/j.1574-6968.2007.00805.x>, 2007.
- 1095 Weigand, M. A., Foriel, J., Barnett, B., Oleynik, S., and Sigman, D. M.: Updates to instrumentation and protocols for isotopic analysis of nitrate by the denitrifier method, *Rapid Commun. Mass Spectrom.*, 30, 1365–1383, <https://doi.org/10.1002/rcm.7570>, 2016.
- Xu, S., Kang, P., and Sun, Y.: A stable isotope approach and its application for identifying nitrate source and transformation process in water, *Environ. Sci. Pollut. Res.*, 23, 1133–1148, <https://doi.org/10.1007/s11356-015-5309-6>, 2016.
- 1100 Yang, J., Heidbuchel, I., Musolff, A., Reinstorf, F., and Fleckenstein, J. H.: Exploring the dynamics of transit times and subsurface mixing in a small agricultural catchment, *Water Resour. Res.*, 54, 2317–2335, <https://doi.org/10.1002/2017WR021896>, 2018.
- Yu, Z., Hu, Y., Gentry, L. E., Yang, W. H., Margenot, A. J., Guan, K., Mitchell, C. A., and Hu, M.: Linking water age, nitrate export regime, and nitrate isotope biogeochemistry in a tile-drained agricultural field, *Water Resour. Res.*, 59, e2023WR034948, <https://doi.org/10.1029/2023WR034948>, 2023.
- 1105