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Isotopes Reveal the Moderating Role of Ammonium on Global Riverine Water Nitrogen Cycling

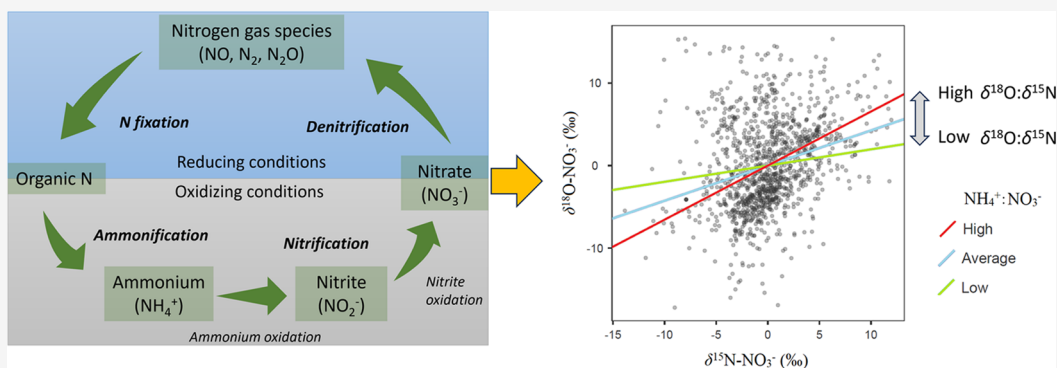Ioannis Matiatos,* Lucilena R. Monteiro, Mathieu Sebilo, David X. Soto, Daren C. Gooddy, and Leonard I. Wassenaar

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ABSTRACT: The relationship between $\delta^{18}O$ and $\delta^{15}N$ in aquatic nitrate (NO_3^-) is used to assess nitrogen (N) cycling, primarily relying on controlled laboratory tests of isotope fractionation from nitrification and denitrification. Nevertheless, laboratory findings frequently contradict the evolution of the nitrate $\delta^{18}O/\delta^{15}N$ ratios observed in natural river systems. We investigated this disparity by using moderated regression modeling, analyzing a global data set ($n = 1303$) of nitrate isotopes encompassing rivers with varying NH_4^+/NO_3^- ratios and $\delta^{18}O-H_2O$ values. First, our analysis revealed that elevated $\delta^{18}O/\delta^{15}N$ ratios (>0.6) were prevalent in rivers with high NH_4^+/NO_3^- ratios, suggesting reducing conditions that could potentially promote denitrification and/or ammonium accumulation. By contrast, lower $\delta^{18}O/\delta^{15}N$ ratios (<0.5) predominated in rivers with low NH_4^+/NO_3^- conditions, suggesting oxidizing conditions favoring increased NH_4^+ removal through nitrification. Second, when $\delta^{18}O-H_2O$ values were low, it resulted in reduced $\delta^{18}O-NO_3^-$ values during nitrification, which in turn lowered the $\delta^{18}O/\delta^{15}N$ ratios. We discovered that the $\delta^{18}O/\delta^{15}N$ ratios in nitrate were elevated in the fall, likely due to predominant processes, such as denitrification, and lower in the winter due to lower $\delta^{18}O-H_2O$ values. This global river assessment suggests a more significant influence of ammonium and the role of water oxygen in riverine N-nutrient isotope cycling than was previously considered.

KEYWORDS: riverine nitrogen, nitrate isotopes, ammonium, denitrification, nitrification, biogeochemical processes, slope ratio analysis

1. INTRODUCTION

Earth's rivers are major aquatic receiving environments of terrestrial nitrogen (N) sources transported via atmospheric deposition, agricultural runoff, hyporheic, or groundwater flow.[1] Protecting the health of Earth's rivers from excess N pollution requires untangling the contributions of the predominant nitrate sources, however, the nonconservative behavior of nitrate in the aquatic N-receiving environment makes source tracing a challenge owing to the coexistence of multiple biogeochemical processes that transform nitrate and other N-forms.[2−14] Microorganisms and biota mediate N transformations in rivers through stepwise oxidation and reduction processes in the water body, the riparian zone, and the water−sediment interface.[15,16]

The complexity of N-cycling in Earth's riverine systems is particularly challenging due to the wide range of influential

factors, including diurnal−seasonal−climatic and productivity changes and other human-induced and N-related inputs into rivers. Notably, the role of ammonium (NH_4^+) and its contribution to nitrate (NO_3^-) levels have not been systematically considered under field riverine system conditions as in well-controlled laboratory experiments. Ammonification of organic matter decay releases ammonium (NH_4^+), but NH_4^+ concentrations often decrease with the increasing amount of suspended organic solids due to adsorption or increased

uptake by plants.[17] In rivers, aerobic oxidation rapidly converts ammonium to nitrite → nitrate, known as nitrification, which often results in riverine NH_4^+ concentrations at or below detection limits and/or decreasing NH_4^+/NO_3^- ratios (e.g., see ref 18). Moreover, NH_4^+ is not always measured in national water quality monitoring programs.

Nitrification is controlled by environmental parameters, such as water pH, dissolved oxygen (DO), water temperature, substrate concentration, and the presence or absence of a nitrifying bacteria population.[19−21] For example, under low pH conditions (pH < 6), ammonium oxidation in water is inhibited. In contrast, nitrite oxidation is strongly inhibited at higher pH values (pH < 8.5), which modulates the rates of nitrification, favoring NH_4^+ accumulation under acidic conditions.[22] In general, low DO concentrations (<2.0 mg/L) in freshwaters inhibit aerobic nitrification due to the reduced ability of nitrifying bacteria to grow, which favors the prevalence of denitrification over nitrification.[23] For example, higher NH_4^+ levels were measured in a eutrophic subtropical stream with constant nitrate loading during the nighttime when DO levels decreased.[24] Under low-oxygen conditions in rivers, organic matter (OM) can contribute to a cascade of N dynamics that promote the removal of nitrate through denitrification or assimilation, all occurring within the same moving water body and at the sediment−porewater interface.[25] Low NH_4^+/NO_3^- ratios have been observed in oxygenated waters of deep lakes due to uptake of NH_4^+ by plants in the photic zone and nitrification to N oxidized forms,[26] whereas, under lower oxygen conditions, NH_4^+ accumulates with respect to NO_3^-, thus increasing NH_4^+/NO_3^- ratios.[27]

In summary, increasing NH_4^+/NO_3^- ratios often reflects low-oxygen and reducing conditions, which could favor denitrification over nitrification. The role of redox conditions on the NH_4^+/NO_3^- ratio is also evidenced by the relationship of the NH_4^+/NO_3^- ratio with gross primary production (GPP). GPP represents the primary production by photosynthetic organisms, including plants and algae, and is influenced by the oxygen availability. Peñarroya et al.[28] showed that NH_4^+/NO_3^- ratios were highest when GPP or oxygen availability was low.

Previous efforts to understand N-cycling in riverine systems were mainly based on controlled laboratory or in situ field chamber incubation experiments (e.g., see ref 29,30). These experiments show variable N and O ($^{15}N/^{14}N$, $^{18}O/^{16}O$) stable isotope fractionation effects, namely, the difference in the $\delta^{15}N$ values in the product and substrate,[31] as expressed by the isotope fractionation factor ε or the apparent fractionation Δ.[31] For example, denitrification can induce an isotope fractionation factor $^{15}\varepsilon$ for $\delta^{15}N$ values between 0 and −40‰, whereas nitrification between −10 and −35‰.[31] Typically, experimental approaches use closed systems to calculate the kinetic isotope effects of the biogeochemical N transformation processes in a one-step process, which implies that no preceding or subsequent steps alter the isotopic composition of the substrate or the product. The investigation of these biogeochemical processes still lacks sufficient explanation, given that nitrate isotope distribution patterns in freshwaters often contradict those appearing in studies dealing with nitrifying and denitrifying bacteria cultures.[32] These differences in the isotopic fractionation effects are partly attributed to challenges in simulating real conditions by using different experimental (organisms, temperature, substrate, isolation) and calculation methods.[32] Particularly, the role of ammonium

(NH_4^+) in understanding the biogeochemical processes in rivers is under investigated[3,27,29] because of its extremely low concentrations in the rivers or lack of routine monitoring, constraining the analysis for N isotopes.

The well-known covariance between $\delta^{15}N−NO_3^-$ and $\delta^{18}O−NO_3^-$, expressed by the $\delta^{18}O/\delta^{15}N$ ratio (or slope, expressed as $\Delta\delta^{18}O/\Delta\delta^{15}N$ in other studies, e.g., see ref 32), is a common proxy used to infer the predominant biogeochemical N transformation process in aquatic environments. A $\delta^{18}O/\delta^{15}N$ ratio slope of 0.5 is typically found in freshwaters undergoing NO_3^- loss via denitrification.[33−36] Conversely, controlled laboratory experiments investigating denitrification and nitrate assimilation consistently find $\delta^{18}O/\delta^{15}N$ ratios closer to 1.[37−41] The $\delta^{18}O/\delta^{15}N$ ratios during assimilation have been found to be similar for marine and freshwater systems.[42] The isotopic fractionation due to ammonification and its impact on $\delta^{18}O/\delta^{15}N$ ratios are not well understood. Experiments in marine particulate matter have shown that ammonification yields a fractionation factor $^{15}\varepsilon$ between −1.43 and −2.3‰.[43] Under anaerobic conditions and depending on the salinity levels, nitrate reduction to ammonium (DNRA) induces nitrate removal and ammonium accumulation yielding $\delta^{18}O/\delta^{15}N$ ratios ranging between 0.86 and 1.02.[37]

Granger and Wankel[32] proposed a one-box process model after finding the $\delta^{18}O/\delta^{15}N$ ratios depended on the relationships between the reduction of NO_3^- to (unstable) NO_2^- and concurrent production of NO_3^- by NO_2^-. Their model examined the $\delta^{18}O/\delta^{15}N$ ratios under aerobic nitrification transitions to anaerobic denitrification. When denitrification dominates over nitrification, the $\delta^{18}O/\delta^{15}N$ ratios are below but close to 1, regardless of the water $\delta^{18}O$ values, but as the influence of nitrification over denitrification increases, the $\delta^{18}O/\delta^{15}N$ ratios decrease and get closer to 0.5 with decreasing $\delta^{18}O−H_2O$ values.[33] The low $\delta^{18}O/\delta^{15}N$ ratios ($\ll 1$) are linked to low values of $\delta^{18}O−H_2O$ given O-isotope exchange with the NO_2^- intermediate and incorporation of ^{18}O depleted water oxygen during NO_2^- oxidation.[44] Low $\delta^{18}O$ values of water thus lead to lower $\delta^{18}O−NO_3^-$ values with nitrification compared to denitrification. The minor differences between $\delta^{18}O−H_2O$ (^{18}O enriched and temporally invariant in seawater) and $\delta^{18}O−NO_3^-$ values in the marine environment were considered responsible for observed $\delta^{18}O/\delta^{15}N$ ratios >1. In contrast, the more considerable difference between these two O-isotopes in freshwater systems triggers $\delta^{18}O/\delta^{15}N$ ratios <1 or lower. The relatively lower $\delta^{18}O/\delta^{15}N$ values in river water systems compared to experimental results were attributed to unresolved influences like river water evaporation, seasonal changes, changes in dissolved oxygen (DO), mixing of N sources, nitrification with higher $\delta^{18}O$ values due to aerobic respiration, and differences in heterotrophic and autotrophic pathways.[33,36,45] On the contrary, the absence of low $\delta^{18}O/\delta^{15}N$ isotope ratios (≈ 0.5) in marine studies was partly attributed to salinity, light, pH, and difference in microbial cultures between fresh and marine waters.[32] Thus, the $\delta^{18}O/\delta^{15}N$ ratio "signal" is considered to be a benchmark distinguishing marine systems from terrestrial systems. Still, these findings associated with laboratory experiments are unconfirmed in riverine system conditions, especially globally.[41]

Here, we investigate riverine $\delta^{18}O/\delta^{15}N$ ratios in field conditions under varying redox conditions via a global nitrate isotope data set spanning a wide range of environmental conditions, using data obtained from the literature. We used

NH_4^+/NO_3^- ratios as a proxy for the redox state and applied a moderated regression analysis (MRA) to investigate its possible influence in nitrification/denitrification conditions. Additionally, to explore the role of $\delta^{18}O-H_2O$ in the $\delta^{18}O/\delta^{15}N$ ratios, we applied the MRA using $\delta^{18}O-H_2O$ values and their relationship with $\delta^{18}O-NO_3^-$ values. Our study thereby revealed the moderating role of NH_4^+ and $\delta^{18}O-H_2O$ in elucidating nitrogen transformation mechanisms and aiding sustainable management and remediation of N-contaminated waters.

2. MATERIALS AND METHODS

2.1. Data Compilation and Processing. We compiled a global nitrate concentration and stable isotope database ($\delta^{15}N$ and $\delta^{18}O$ of NO_3^- and $\delta^{18}O$ of H_2O) of river waters from Matiatos et al.[45] ($n = 1303$, $n_{rivers} = 58$ rivers), complemented with NH_4^+ concentration data obtained from the same literature sources cited within. Wherever NH_4^+ concentrations were unreported in the publications, we assumed that they were not measured or below detection. Most river waters were sampled monthly and analyzed with variable techniques for $\delta^{18}O$ and $\delta^{15}N$ of NO_3^- (e.g., see refs 46−48). The riverine nitrate isotope database covered the time from 1999−2021 and spanned a broad latitudinal gradient (from 67° N to 38° S) and a longitudinal range (from 145° E to 123° W), including a wide range of ecozone conditions. The data set was mainly represented by Köppen temperate climatic zone rivers (C type[49]).

We explored global variations in the observed $\delta^{18}O/\delta^{15}N$ ratios by linking the NH_4^+/NO_3^- ratios to redox conditions and oxygen availability. Mainly, high NH_4^+/NO_3^- ratios were related to reduced oxygen conditions, whereas low or zero NH_4^+/NO_3^- ratios were associated with the increasing influence of aerobic conditions. Although DO levels would be a strong argument to support this hypothesis, dissolved oxygen values were only available in <10% of the river data sets. We considered the moderating effects of NH_4^+/NO_3^- concentration ratios influencing the relationship between $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- and thus the $\delta^{18}O/\delta^{15}N$ ratio by applying a moderated regression analysis. Moderated regression analysis tests whether the moderator changes the strength of the relationship between the predictor ($\delta^{15}N-NO_3^-$) and the dependent variable ($\delta^{18}O-NO_3^-$).[50] In MRA, interaction terms (e.g., $\delta^{15}N-NO_3^- x NH_4^+/NO_3^-$) created by multiplying two (or more) variables together are used as a proxy to explore whether the relationship between the independent variable(s) and the dependent variable differs under different conditions or levels of a third variable. The presence or absence of an interaction can significantly affect the interpretation of the MRA. For example, if there was no interaction, it suggests that the relationship between the independent variable(s) and the dependent variable was consistent across all levels of the moderator. On the other hand, if there is an interaction, it reveals that the relationship changes or varies based on different levels of the moderator. To check for possible bias of the unreported NH_4^+ concentration assumption on the final result, we created a subset of data ($n = 407$), including only reported NH_4^+ concentrations ($NH_4^+ > 0$), and applied moderated regression analysis.

To examine the role of water $\delta^{18}O$ on the $\delta^{18}O/\delta^{15}N$ ratio of nitrate, we applied the MRA by using the following moderators: (i) $\delta^{18}O-H_2O$, (ii) the absolute difference ($\Delta\delta^{18}O$) between the $\delta^{18}O-NO_3^-$ and $\delta^{18}O-H_2O$ values,

and (iii) the difference ($\Delta\delta^{18}O-NO_3^-$) between the measured $\delta^{18}O-NO_3^-$ and the theoretical $\delta^{18}O-NO_{3(th)}^-$ values produced during nitrification by using the following simple isotope mass balance equation[44]

$$\delta^{18}O\text{-}NO_{3(th)}^- = 2/3 \times \delta^{18}O\text{-}H_2O + 1/3 \times \delta^{18}O\text{-}O_2 \tag{1}$$

Only positive $\Delta\delta^{18}O-NO_3^-$ values ($n = 421$) were considered given that positive deviations from the nitrification line reflect denitrification processes.[51] It should be noted that $\delta^{18}O-H_2O$ represents riverine values determined on the same water with the nitrate isotopes and is expected to be controlled mostly by water sources and evaporation processes and less by atmospheric $\delta^{18}O-NO_3^-$ given the fast equilibration of atmospheric nitrate with riverine water.[33] For $\delta^{18}O-O_2$, a constant value of +24.2‰ was considered.[52]

Mean-centered scores were used in the regression to minimize the problem of multicollinearity.[53] A simple slope analysis was used (least-squares linear regression model) to explore the impact of the moderators on the relationship between $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- by examining the unstandardized regression slope at lower (mean −1 SD), mean, and higher (mean +1 SD) levels.[54] Before applying the MRA, we examined the data for variable intercorrelation using Spearman's correlation and a level of significance p-value <0.05.[55] To test whether the individual slopes of the models are different, a t-value for the difference in the two slopes was computed and then converted to p-value.[56] To investigate the influence of seasonality on the $\delta^{18}O/\delta^{15}N$ ratios, we used riverine data from temperate ecozones (C type) given that seasonality is far stronger in this zone compared with the others. For this analysis, a generalized linear model (GLM) was applied using $\delta^{18}O-NO_3^-$ as the dependent variable and $\delta^{15}N-NO_3^-$ and NH_4^+/NO_3^- as the independent variables. Seasonality was introduced as a categorical predictor after classification of seasons as follows: autumn = 1, winter = 2, spring = 3, and summer = 4. Seasons were identified separately for each hemisphere (N vs S) using the reported sampling date (month) to ensure consistency. The interactions among the independent variables were also considered.

The $^{15}N/^{14}N$ and $^{18}O/^{16}O$ ratios of NO_3^- were reported in the usual δ notation in per mil (‰), where $\delta = (R_{sample}/R_{AIR \text{ or } VSMOW} - 1)$ and R is the ratio of $^{15}N/^{14}N$ or $^{18}O/^{16}O$ in the sample and in the international standard: atmospheric air (AIR) or Vienna standard mean ocean water (VSMOW), respectively. All statistical tests used jamovi v. 2.3 and R v.4.1.[57,58]

3. RESULTS AND DISCUSSION

3.1. Moderating Role of NH_4^+/NO_3^- on $\delta^{18}O/\delta^{15}N$ Ratios. The riverine $\delta^{15}N-NO_3^-$ values were significantly correlated with the $\delta^{18}O-NO_3^-$ values ($r \approx 0.3$) and the NH_4^+/NO_3^- ratios ($r \approx 0.1$) but not with the interaction $\delta^{15}N-NO_3^- \times NH_4^+/NO_3^-$ (Table S1). The $\delta^{18}O-NO_3^-$ values were significantly and negatively correlated with the NH_4^+/NO_3^- ratios and the interaction $\delta^{15}N-NO_3^- \times NH_4^+/NO_3^-$ ($r \approx 0.3$). The NH_4^+/NO_3^- ratios were associated with the interaction $\delta^{15}N-NO_3^- \times NH_4^+/NO_3^-$ ($r \approx 1.0$). It should be noted that the correlation of the interaction term with other variables is not necessarily evidence for moderation, as this requires the application of the MRA.[59] The NH_4^+/NO_3^- ratio, the $\delta^{15}N-NO_3^-$, and the $NH_4^+/NO_3^- \times \delta^{15}N-$
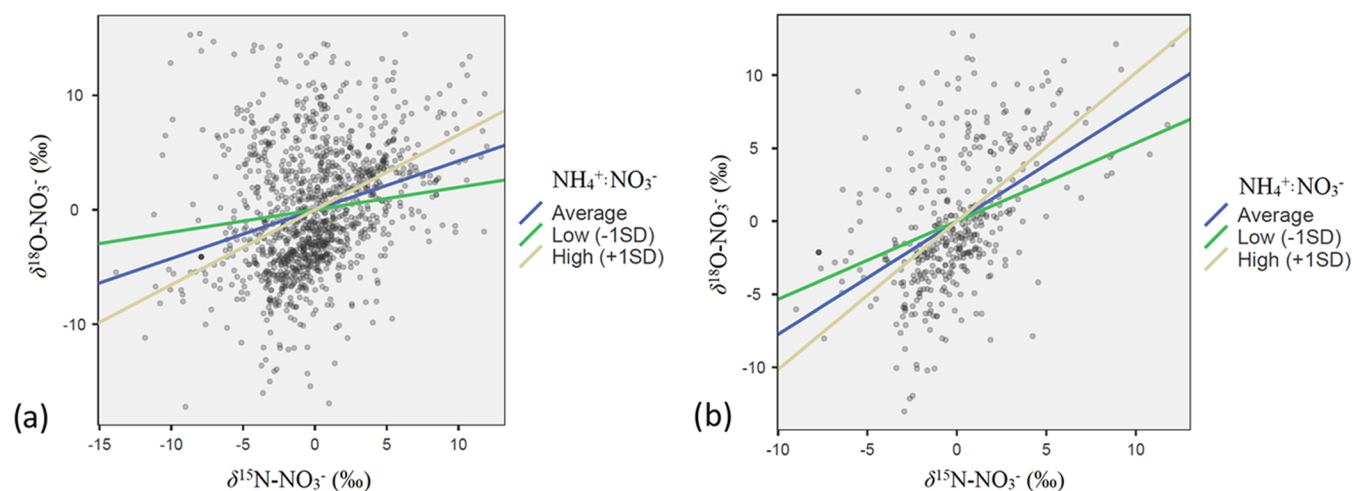
Figure 1. Plot depicting the moderating effect of NH_4^+/NO_3^- ratio on the $\delta^{18}O/\delta^{15}N$ ratio using (a) the whole data set ($N = 1.303$) and (b) the subset of reported $NH_4^+ \neq 0$ values ($N = 407$). The color legend refers to the levels of the NH_4^+/NO_3^- ratio based on the moderated regression analysis.

NO_3^- interaction were significant ($p < 0.05$) parameters in explaining the $\delta^{18}O-NO_3^-$ variance (Table S2).

The analysis (Table S3 and Figure 1a) revealed that the slope (as expressed by the $\delta^{18}O/\delta^{15}N$ ratio) estimate was high (0.655) when NH_4^+/NO_3^- ratios were high, whereas low NH_4^+/NO_3^- levels produced a more downward slope estimate (0.197), with significant difference ($p < 0.05$) between the high and low slope estimates. Similarly, using the subset of $NH_4^+ \neq 0$ data, the MRA revealed a high slope estimate (1.015) when the NH_4^+/NO_3^- ratios were high and lower slope estimates (0.535) when the NH_4^+/NO_3^- levels were low (Tables S4 and S5 and Figure 1b).

The MRA provided evidence for the moderating role of NH_4^+/NO_3^- concentration ratios on the relationship between $\delta^{18}O-NO_3^-$ and $\delta^{15}N-NO_3^-$ and consequently on the $\delta^{18}O/\delta^{15}N$ ratio. The high NH_4^+/NO_3^- ratio generally represents reducing or anaerobic conditions. Organic matter decomposition and anaerobic conditions favored at the interface of river water with the sediment and the suspended particles can explain NH_4^+ accumulation over NO_3^- and a possible dominance of denitrification (and/or DNRA) over nitrification. The $\delta^{18}O/\delta^{15}N$ ratio increased as the NH_4^+/NO_3^- ratio increased, bringing the slope closer to one, as observed in controlled laboratory incubation experiments. On the contrary, the low NH_4^+/NO_3^- levels represent aerobic or oxidizing riverine conditions, where NH_4^+ is transformed to NO_3^- through nitrification, lowering the $\delta^{18}O/\delta^{15}N$ ratios. This suggests that despite low to undetectable NH_4^+ concentrations in most riverine systems, NH_4^+ that was oxidized to NO_2^- can be revealed by the $\delta^{18}O/\delta^{15}N$ decrease due to nitrification. Apart from atmospheric nitrate and nitrate synthetic fertilizers, nitrate primarily originates from nitrification of NH_4^+ and organic-based sources (e.g., NH_4^+ in fertilizers, wastewaters, and manure). However, it should be noted that nitrification is not the only source of nitrate in rivers. Atmospheric nitrate, often with an $\delta^{17}O$ anomaly, is expected to be a significant source of nitrate in lakes and pristine ecosystems.[60] On the other hand, as of 2021, urea and NPK (nitrogen–phosphorus–potassium) are among the world's most common and widely used synthetic nitrogen fertilizers in the agricultural sector and less the nitrate synthetic fertilizers.[61] Additionally, ammonium accumulation under equilibrium or steady-state

conditions between NO_2^- and H_2O is possible when the $\delta^{15}N-NO_3^-$ values produced by nitrification converge with those generated from denitrification;[32] however, equilibrium conditions are challenging to find in natural freshwater settings given that they are open and dynamic systems.

3.2. Moderating Role of $\delta^{18}O-H_2O$ on $\delta^{18}O/\delta^{15}N$ Ratios. The sensitivity of the $\delta^{18}O/\delta^{15}N$ ratios to $\delta^{18}O-H_2O$ was examined by using $\delta^{18}O-H_2O$, $\Delta\delta^{18}O$, and $\Delta\delta^{18}O-NO_3^-$ as moderators in the MRA (see Methods section). The $\delta^{15}N-NO_3^-$ values were correlated significantly and positively with the $\delta^{18}O-NO_3^-$ and $\delta^{18}O-H_2O$ values ($r > 0.25$) and the $\delta^{15}N-NO_3^- \times \Delta\delta^{18}O$ and $\delta^{15}N-NO_3^- \times \Delta\delta^{18}O-NO_3^-$ interactions ($r > 0.5$) and negatively with $\delta^{15}N-NO_3^- \times \delta^{18}O-H_2O$ ($r < -0.5$) (Table S6). The $\delta^{18}O-NO_3^-$ values were correlated positively with the $\Delta\delta^{18}O$ and $\Delta\delta^{18}O-NO_3^-$ values and the related interactions ($r > 0.6$) but less with $\delta^{18}O-H_2O$ values ($r > 0.3$). The $\delta^{18}O-H_2O$, $\delta^{15}N-NO_3^-$, and $\delta^{18}O-H_2O \times \delta^{15}N-NO_3^-$ interactions were significant in explaining the variance of $\delta^{18}O-NO_3^-$ (Table S7).

The simple slope analysis (Table S8 and Figure 2a) showed that the slope (or $\delta^{18}O/\delta^{15}N$ ratio) estimate was higher (0.334) when $\delta^{18}O-H_2O$ values were high, whereas low $\delta^{18}O-H_2O$ values induced a more downward slope estimate (0.165). The difference between the high and low slope estimates was significant ($p < 0.05$). The results of the MRA when using $\Delta\delta^{18}O$ and $\Delta\delta^{18}O-NO_3^-$ as moderators showed that their effect on $\delta^{18}O-NO_3^-$ was not significant (Tables S9, S10, S11, S12 and Figures S1, S2).

The MRA revealed the influence of $\delta^{18}O$ of ambient H_2O on the $\delta^{18}O/\delta^{15}N$ ratios of nitrate. When $\delta^{18}O-H_2O$ was used as a moderator, the $\delta^{18}O/\delta^{15}N$ ratios were lower at more negative $\delta^{18}O-H_2O$ levels. This finding was consistent with the Granger and Wankel experiments,[32] who showed a decrease in the $\delta^{18}O/\delta^{15}N$ ratio of nitrate with decreasing $\delta^{18}O-H_2O$ values. Given the relationship of $\delta^{18}O-NO_3^-$ with $\delta^{18}O-H_2O$ (eq 1), low $\delta^{18}O-H_2O$ values contribute to low $\delta^{18}O$ values for nitrified NO_3^-, leading to a decrease in the $\delta^{18}O/\delta^{15}N$ ratios. When the initial $\delta^{18}O-NO_3^-$ is closer to $\delta^{18}O$ of H_2O, the $\delta^{18}O-NO_3^-$ produced during nitrification is higher than that removed by denitrification, which increases the $\delta^{18}O/\delta^{15}N$ ratios. On the contrary, when the oxygen isotope difference between the initial $\delta^{18}O-NO_3^-$ and $\delta^{18}O-H_2O$ is higher,
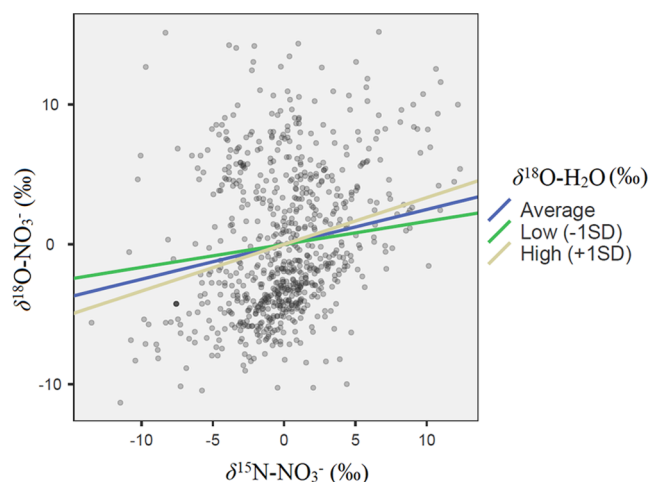
Figure 2. Plot depicting the moderating effect of $\delta^{18}O-H_2O$ values on the $\delta^{18}O/\delta^{15}N$ ratio. The color legend refers to the levels of $\delta^{18}O-H_2O$ based on the moderated regression analysis.

difference between spring and winter NH_4^+/NO_3^- ratio was statistically significant ($p < 0.05$).

The relationship between $\delta^{15}N$ and $\delta^{18}O$ of nitrate was significant ($p < 0.05$) for all seasons (Table S14, Figure 3). In

Figure 3. Seasonal evolution of $\delta^{18}O/\delta^{15}N$ isotope ratios in temperate rivers. The $\delta^{18}O/\delta^{15}N$ isotope ratio is the slope of the regression line in each $\delta^{15}N-NO_3^-$ versus $\delta^{18}O-NO_3^-$ plot.

denitrification decreases the value of $\delta^{18}O-NO_3^-$ that is equal or greater than that added by nitrification, contributing to a decrease in the $\delta^{18}O/\delta^{15}N$ ratios.[32] In freshwater systems, the difference between $\delta^{18}O-NO_3^-$ and $\delta^{18}O$ values of H_2O can be considerable, which promotes lower $\delta^{18}O/\delta^{15}N$ ratios of nitrate compared to marine systems, where the oxygen isotope difference is more minor.

When the influence of the absolute difference ($\Delta\delta^{18}O$) between $\delta^{18}O-NO_3^-$ and $\delta^{18}O-H_2O$ was considered, surprisingly, it was not a significant moderator, even though $\delta^{18}O-H_2O$ data set included a wide range of δ values. This finding is probably because of the $\delta^{18}O-H_2O$ influence during the oxidation of NH_4^+ to NO_2^- and the oxidation of NO_2^- to NO_3^- and the extent of equilibrium between the NO_2^- intermediate and H_2O.[62,63] Similarly, the deviation ($\Delta\delta^{18}O-NO_3^-$) of the observed $\delta^{18}O-NO_3^-$ from the theoretical nitrification $\delta^{18}O-NO_{3(th)}^-$ values was not a significant moderator for the relationship between $\delta^{18}O$ and $\delta^{15}N$ of NO_3^-. This is because the deviations from the theoretical nitrification value of $\delta^{18}O-NO_3^-$ in rivers can occur from mixing processes and also from the variation of $\delta^{18}O-O_2$ driven by aquatic productivity[52] in addition to the $\delta^{18}O-H_2O$ value. Snider et al.[63] highlighted the complexity of $\delta^{18}O-NO_3^-$ formation in aerobic environments via nitrification, which is not accurately described by a simple mass balance model (eq 1). Thus, the modulation of $\Delta\delta^{18}O-NO_3^-$ follows a complex pattern, integrating several contributors together. The assessment of each original variation source (water, O_2, and/or N source mixed) should be better understood in a similar analysis.

3.3. Seasonal Variations of $\delta^{18}O/\delta^{15}N$ Ratios in Temperate Zone Rivers. The results of the GLM exploring the seasonal influence on $\delta^{18}O/\delta^{15}N$ ratios are summarized in Table S13, where the likelihood ratios represent the contributions of each variable and their interactions in the model. The results showed that $\delta^{15}N-NO_3^-$ and NH_4^+/NO_3^- ratios significantly affected the model, whereas seasonality had no significant effect. However, the impact of the season was significant in the interaction with $\delta^{15}N-NO_3^-$ and NH_4^+/NO_3^- ratio values. In spring, the mean NH_4^+/NO_3^- ratio was highest (1.22), followed by autumn and summer (~0.99 and ~0.57, respectively) and winter (0.16). However, only the
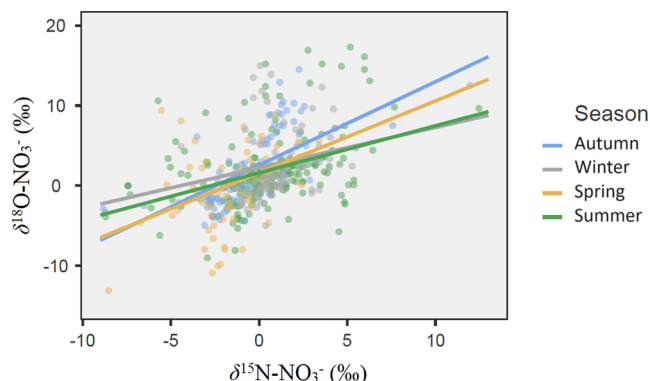
winter and summer, the $\delta^{18}O/\delta^{15}N$ ratio estimate was between 0.5 and 0.6. with $r = 0.2$ and 0.4, respectively. In autumn and spring, the $\delta^{18}O/\delta^{15}N$ ratio estimate was higher (>1.0 and 0.9, respectively) with $r = 0.7$ and 0.4, respectively. Despite the slight differences between the seasons, it is worth noting that autumn and spring showed significantly higher $\delta^{18}O/\delta^{15}N$ ratios in comparison to winter and summer. In autumn and spring, the high $\delta^{18}O/\delta^{15}N$ ratios and the high NH_4^+/NO_3^- ratios could indicate reducing or anaerobic conditions, favoring denitrification prevalence over nitrification. Low GPP conditions have been recorded in summer and autumn in temperate climates (e.g., see ref 28). Dissimilatory nitrate reduction to ammonium (DNRA) is also favored under reducing conditions inducing high $\delta^{18}O/\delta^{15}N$ and NH_4^+/NO_3^- ratios.[37] Spring also favors the uptake of nutrients by plants, which means that the increased $\delta^{18}O/\delta^{15}N$ ratios can be partly attributed to the isotope fractionation of nitrate isotopes due to assimilation.[33] Other mixing processes during spring, e.g., mixing with waters of high $\delta^{18}O$ and low $\delta^{15}N$ values during periods of high discharge,[10,64,65] typically in the spring freshet for temperate rivers could partly control the $\delta^{18}O/\delta^{15}N$ ratios.

In the summer, the $\delta^{18}O/\delta^{15}N$ ratio estimate could indicate denitrification predominance over nitrification due to decreased DO levels (from higher water temperature and diel productivity) and river discharge. However, N fluxes in rivers in summer may be biased by changes in the proportion of baseflow conditions, namely, the more significant influence of groundwater discharge or seepage with cycled N transported via the unsaturated zone.[66] This means that groundwater-fed rivers are usually more depleted in ^{18}O (of H_2O) than summertime precipitation.

During winter in temperate rivers, algal growth is limited with a predominance of aquatic plant senescence and organic matter mineralization and changes in oxidation–reduction potential under typically elevated DO conditions.[67] This is because colder water temperatures can hold more dissolved oxygen compared to warmer temperatures. Additionally, increased water flow in the winter can help aerate the water and elevate DO levels. However, the disposal of wastes (e.g.,

industrial wastes, stormwater, sewage effluents) in rivers can induce decreasing of DO concentration.[68] Similarly, ice cover can limit the exchange of oxygen between the river water and the atmosphere and potentially decrease DO levels.[68,69] The winter slope estimates of ~0.5 and the low NH_4^+/NO_3^- ratios could be related to nitrification predominance over denitrification. Additionally, lowering the slope estimate could be explained by the lower $\delta^{18}O$ of river H_2O during winter, which decreased the $\delta^{18}O/\delta^{15}N$ ratio, as explained above.

Identifying seasonal patterns of the $\delta^{18}O/\delta^{15}N$ ratios required aggregating diverse nitrate sources as reflected in the $\delta^{15}N-NO_3^-$ values, typically observed at the catchment scale or complicated by seasonal variability. For example, Matiatos et al.[45] showed that the high $\delta^{15}N-NO_3^-$ values in winter indicate degradable organic-related N sources, which are better preserved at low water temperatures. On the contrary, the low $\delta^{15}N-NO_3^-$ values during spring can also partly reflect the influence of applying synthetic fertilizers at the start of the growing season and its runoff.[10]

4. CONCLUSIONS

The confounding concurrence of biogeochemical processes affecting water nitrate, nitrite, and ammonium content (e.g., nitrification, denitrification), along with multiple potential N sources, challenges a simplistic assessment for remediation of nitrate (NO_3^-) water pollution in Earth's rivers, suggesting that nitrate isotope tracers should be used with caution as source or process indicators. Our understanding of the relative roles of various N-cycling processes remains surprisingly poor, particularly concerning the interpretation of nitrate isotope patterns observed in rivers. These often appear to be at odds with the findings from controlled experimental laboratory studies of nitrifying and denitrifying bacteria cultures. The role of NH_4^+ in N-cycling has not yet been widely considered in aquatic nitrate isotope systematics primarily due to the "invisibility" of NH_4^+ in most aquatic systems once it has been oxidized, either by NH_4^+ absence or the lack of its routine measurement. Accordingly, we recommend that NH_4^+ assays (and, if possible its $\delta^{15}N$) should be an essential measurement to improve aquatic isotope studies of nitrate along with assays of $\delta^{18}O$ of river water.

Our assessment showed that NH_4^+/NO_3^- concentration ratios could be used as a proxy of oxidizing/aerobic and reducing/anaerobic conditions. In particular, high NH_4^+/NO_3^- concentration ratios were linked to reducing and low-oxygen availability conditions, which could favor denitrification processes. The high $\delta^{18}O/\delta^{15}N$ ratios produced under high NH_4^+/NO_3^- concentration ratios coincided with the dominance of denitrification. On the contrary, low NH_4^+/NO_3^- concentration ratios were associated with increased nitrification influence over denitrification, which induced lower $\delta^{18}O/\delta^{15}N$ ratios.

However, as the nitrification influence increases over denitrification, the $\delta^{18}O/\delta^{15}N$ ratios are regulated mainly by $\delta^{18}O$ of H_2O. The moderating role of $\delta^{18}O$ of H_2O on decreasing $\delta^{18}O/\delta^{15}N$ ratios in river waters thus confirmed the previous findings of Granger and Wankel.[32] In temperate rivers, the $\delta^{18}O/\delta^{15}N$ ratios were higher in the autumn, which could be related to denitrification processes. In early autumn, water temperatures can still be relatively warm from the preceding summer, leading to lower DO concentrations (e.g., see ref 70). Additionally, in autumn in temperate climates, the shedding of leaves and vegetation can contribute organic

matter to rivers. The decomposition of this organic material consumes oxygen, leading to increasing DOC (dissolved organic carbon) and decreasing DO levels in water (e.g., see ref 71). In winter, the low $\delta^{18}O/\delta^{15}N$ ratios were partly attributed to the influence of lower $\delta^{18}O$ of H_2O on $\delta^{18}O-NO_3^-$ due to colder temperatures.[72]

The nonmoderating role of $\Delta\delta^{18}O$ could be indicative of nonfull equilibration between NO_2^- oxygen isotopes with water and a possible influence of $\delta^{18}O-H_2O$ and O_2 in a more complex way than a simple mass balance model (eq 1). In the first case, the O-isotope exchange of NO_2^- with H_2O before being oxidized to NO_3^- can potentially increase $\delta^{18}O-NO_2^-$ higher than the $\delta^{18}O-H_2O$ values and eventually increase $\delta^{18}O-NO_3^-$ values.[29,32,62,63] This process is pH dependent in such a way that it is rapid in low pH levels, whereas at near-neutral pH values (6 and 8), the exchange is typically <50%, depending on the temperature.[63] In the second case, the nonmoderating role of $\Delta\delta^{18}O-NO_3^-$ can be due to processes affecting the isotopic composition of O_2 and mixing processes with atmospheric deposition that need to be considered. The $\delta^{18}O$ of O_2 is heavily dependent on seasonal aquatic productivity and can vary on a diel basis, making the assumption of a constant $\delta^{18}O$ of O_2 or in equilibrium with air (+23.5 to + 24.2‰) very simplistic.[63] For example, photosynthesis in rivers, which typically occurs in the well-mixed water column and by benthic autotrophs (with light availability), induces lower $\delta^{18}O$ values (as low as +3.4‰), particularly during the growing season.[52] Also, when the contribution of atmospheric NO_3^- deposition is relatively high, especially during stormwater runoff (e.g., see ref 73), the $\delta^{18}O-NO_3^-$ can be much affected compared to differences of $\delta^{18}O$ of H_2O in precipitation and river water, which could tilt the $\delta^{18}O/\delta^{15}N$ ratio.

Interpretations of nitrate isotopes may be further complicated, where nitrogen undergoes biogeochemical transformations like in rivers. Thus, the $\delta^{18}O/\delta^{15}N$ ratios presented here reflect a cumulative effect of biogeochemical and mixing processes influenced by variable nitrate sources, which cannot be disentangled using only nitrate stable isotopes. We identified several distinctive global biogeochemical patterns of nitrate in rivers, recognizing that this attempt required aggregating many potential diverse nitrate sources on a catchment and seasonal scale. Although nitrate isotope techniques are helpful to assess the origin of nitrogen pollution in aquatic systems, their interpretations will be complicated when nitrogen undergoes many biogeochemical transformations.

Overall, our assessment highlighted the transient role of "invisible" (e.g., oxidized) ammonium (NH_4^+) in the biogeochemical processes of rivers, which so far has not been recognized or investigated. Due to the simultaneous presence of ammonium as an important substrate and a product of biogeochemical reactions due to its rapid oxidation in aerobic water, the interpretation of nitrogen isotopes of NH_4^+ has proven challenging.[74] However, the importance of ammonium suggests that NH_4^+, pH, and DO concentrations and, ideally, the $\delta^{18}O$ of both H_2O and O_2 data should be systematically collected and reported even when NH_4^+ is expected to be below the detection limit.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.3c00605.

Set of Figures and Tables related to moderated regression analysis and other statistical techniques presented in the main text (PDF)

List of references that were used to retrieve the global data set of NH_4^+ concentrations in river waters (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Ioannis Matiatos − *Institute of Marine Biological Resources and Inland Waters, Hellenic Centre for Marine Research, 19013 Anavissos Attikis, Greece;* ⓞ orcid.org/0000-0002-0395-2678; Email: i.matiatos@hcmr.gr

Authors

Lucilena R. Monteiro − *Instituto de Pesquisas Energéticas e Nucleares, IPEN/CNEN, 05508-000 São Paulo − SP, Brazil*

Mathieu Sebilo − *Sorbonne Université, CNRS, IEES, F-75005 Paris, France*

David X. Soto − *Leibniz Institute for Zoo and Wildlife Research (IZW), 10315 Berlin, Germany;* ⓞ orcid.org/0000-0003-4210-293X

Daren C. Gooddy − *British Geological Survey, Wallingford, Oxfordshire OX10 8BB, United Kingdom;* ⓞ orcid.org/0000-0002-6015-1332

Leonard I. Wassenaar − *WasserCluster Lunz Biological Station, 3293 Lunz am See, Austria*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsestwater.3c00605

Author Contributions

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