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RESEARCH ARTICLE

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Challenges in measuring nitrogen isotope signatures in inorganic nitrogen forms: An interlaboratory comparison of three common measurement approaches

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Rationale: Stable isotope approaches are increasingly applied to better understand the cycling of inorganic nitrogen (N_i) forms, key limiting nutrients in terrestrial and aquatic ecosystems. A systematic comparison of the accuracy and precision of the most commonly used methods to analyze $\delta^{15}N$ in NO_3^- and NH_4^+ and interlaboratory comparison tests to evaluate the comparability of isotope results between laboratories are, however, still lacking.

Methods: Here, we conducted an interlaboratory comparison involving 10 European laboratories to compare different methods and laboratory performance to measure $\delta^{15}N$ in NO_3^- and NH_4^+. The approaches tested were (a) microdiffusion (MD), (b) chemical conversion (CM), which transforms N_i to either N_2O (CM-N_2O) or N_2 (CM-N_2), and (c) the denitrifier (DN) methods.

Results: The study showed that standards in their single forms were reasonably replicated by the different methods and laboratories, with laboratories applying CM-N_2O performing superior for both NO_3^- and NH_4^+, followed by DN. Laboratories using MD significantly underestimated the "true" values due to incomplete recovery and also those using CM-N_2 showed issues with isotope fractionation. Most methods and laboratories underestimated the at%^{15}N of N_i of labeled standards in their single forms, but relative errors were within maximal 6% deviation from the real value and therefore acceptable. The results showed further that MD is strongly biased by nonspecificity. The results of the environmental samples were generally highly variable, with standard deviations (SD) of up to ± 8.4‰ for NO_3^- and ± 32.9‰ for NH_4^+; SDs within laboratories were found to be considerably lower

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(on average 3.1‰). The variability could not be connected to any single factor but next to errors due to blank contamination, isotope normalization, and fractionation, and also matrix effects and analytical errors have to be considered.

Conclusions: The inconsistency among all methods and laboratories raises concern about reported $\delta^{15}N$ values particularly from environmental samples.

1 | INTRODUCTION

Nitrogen (N), a key limiting nutrient in terrestrial and aquatic ecosystems, is predominantly taken up by plants and phytoplankton in its inorganic forms, nitrate (NO_3^-) and ammonium (NH_4^+). It is very important in regulating ecosystem productivity and carbon (C) sequestration[1,2] and plays a key role in the internal N cycling in natural and managed systems. To increase yields of cultured plants or organisms, management practices, including N fertilizer application, have been developed. However, the effects of increasing fertilizer application as well as increasing anthropogenic N deposition are far-reaching:[3] excess inorganic N (N_i) imposes serious environmental risks, by contaminating groundwaters,[4] causing eutrophication of coastal areas,[4] promoting soil acidification,[5] and increasing emissions of reactive N-gases, such as nitrous oxide (N_2O), nitric oxide (NO), and ammonia (NH_3).[6,7]

The negative effects of excess N on water and air quality and human health have intensified the research efforts to better understand and predict the role and fate of N_i in ecosystems in recent years.[6,8–10] Traditional methods (e.g., wet chemical methods, spectroscopic methods) quantify concentration changes in NO_3^- and NH_4 over time to determine net nitrification and mineralization rates. However, these methods have substantial limitations as they do not assess process rates of these bioavailable N forms, and they do not allow to partition source pools of N species.[11] Therefore, stable isotope approaches based on the natural abundance (NA) or experimental isotopic enrichment of ^{15}N (and ^{18}O) in NO_3^- and NH_4^+ have become popular. They are increasingly applied to track gross transformations of N, to partition N cycling pathways, and to determine N sources and sinks.[12–15] Inferences of rate constants and source contributions from both approaches rely on precise measurements of the isotopic composition ($\delta^{15}N$, at% ^{15}N) of N_i.

To date, three commonly used approaches exist to analyze the ^{15}N isotopic composition of NO_3^- and NH_4^+: (a) microdiffusion (MD), (b) chemical conversion (CM), and (c) biological conversion, that is, denitrifier (DN) methods (Figure S1 [supporting information]). In addition, there are protocols that combine parts of several of the afore-mentioned methods.[16] In general, all methods are based on the selective separation and purification of N_i from the sample matrix. The MD method relies on a pH increase of the sample to convert NH_4^+ from solution into gaseous NH_3 that is collected on acidified filters. The isotopic signature of NH_4-N is measured using elemental analyzer-isotope ratio mass spectrometry (EA-IRMS).[17–20] The MD method further enables the analysis of NO_3, which must be reduced first to NH_4 using Devarda's alloy.[21] In the CM methods, NH_4^+ or NO_3^- is chemically converted to N_2O or N_2, which are thereafter analyzed for their isotopic composition using gas chromatography (GC)-IRMS, purge-and-trap (PT)-IRMS,[16,22–24] or laser spectrometric analysis.[22] Chemical approaches also comprise ion exchange resins for collection of NO_3^- and NH_4^+, followed by precipitation as silver, potassium, or barium salts for NO_3^- or conversion to N_2 for NO_3^- and NH_4^+.[23–26] In the DN method, NO_3^- is converted to N_2O by a denitrifying bacterial pure culture that lacks the N_2O reductase enzyme,[27,28] and the isotope composition of N_2O is then analyzed with the techniques mentioned earlier for the CM method. Generally, in the case of labeled samples, scanning mass spectrometers (e.g., quadrupole mass spectrometers[29] or membrane inlet mass spectrometers[30,31]) can also be used for the analysis of isotope ratios in gaseous reaction products. In this interlaboratory comparison, however, only IRMS-based methods are compared, which are most commonly adopted.

Although the potential bias of each individual method has been examined,[32–35] a systematic comparison of the accuracy and precision of the most commonly used IRMS-based methods is still lacking. Moreover, an interlaboratory comparison is lacking where all the possible sources of variation (from sample pretreatment to isotope analysis, including standardization procedures, instrumentation, and data processing) are considered and the validity of the results from laboratories using the same or different method, thus laboratory performance, is assessed. We therefore conducted an interlaboratory comparison on a set of standards and environmental samples containing NO_3^- and/or NH_4^+. All materials were centrally prepared and sent to 10 laboratories, each of which employed at least one of the three recognized techniques for the analysis of isotope ratios ($^{15}N/^{14}N$) in NO_3^- and/or NH_4^+. The main aim was to evaluate the performance and practicability of the three techniques relative to one another and to investigate the intercomparability of the results from different laboratories. This knowledge is needed when designing and interpreting experiments that study the fate, origin, and transformation of N_i based on ^{15}N in NO_3^- and NH_4^+. Accurate results on $\delta^{15}N$ of N_i, which are intercomparable between laboratories, are also needed when laboratories are providing data to a common database, when data synthesis is conducted or when several laboratories are cooperating in joint field surveys. Based on the results, the strengths and weaknesses of each method are discussed, and guidelines are provided, which should aid in choosing the best methods for specific applications and research needs.

2 | EXPERIMENTAL

2.1 | Participating laboratories and organizations in the interlaboratory comparison

The participants in the interlaboratory comparison comprised 10 well-established laboratories associated with universities, nonuniversity research institutions, and commercial laboratories with mostly long experience in analyzing $\delta^{15}N$ of N_i. Each laboratory used its routine methods to analyze standards and environmental samples. Reported results were assigned lab codes (L1 to L16) to ensure confidentiality regarding the identity of the laboratories (on request, lab IDs and original data are available) and were grouped by method type (CM-N_2O, CM-N_2, MD, and DN; Tables S1A and S1B [supporting information]; Figure S1 [supporting information]). As some laboratories employed more than one method, the total number of lab codes is greater than the number of participants. The participants were given basic information on the origin of the environmental samples, including the approximate range of $\delta^{15}N$ values of the standards, to allow for proper referencing (Tables S2 and S3 [supporting information]), but neither actual $\delta^{15}N$ values nor results of other test participants were distributed among the participating laboratories. All standards and environmental samples were centrally prepared at the University of Vienna and dispatched to the participating laboratories immediately after preparation. A range of isotope standards with different $\delta^{15}N$ values was prepared (from −3.04 to 28.1‰), and, in addition to NA, labeled standards (LA) were distributed as well (Table S2 [supporting information]). Participants were asked to deep-freeze the received samples until analysis (mostly done within 6 months after receipt of samples) and to use their routine, in-house methods, and calibration/normalization procedures to provide the raw and final results, along with a clear description of the method used. Each laboratory carried out preparatory steps to isolate the N forms according to their routine methods and analyzed the standards/samples using IRMS (Figure S1 [supporting information]). Therefore, this interlaboratory comparison examines the variability of the whole method chain, from the isolation to the analysis of $\delta^{15}N$ of N_i forms, to the instrumentation used, standardization methods applied, laboratory conditions prevailing, experience level of operators, and the data processing implemented. Statistical analysis, evaluation of the data, and further corrections and calculations (e.g., recovery rates) were carried out either by the individual laboratories or by the main author.

2.2 | Preparation of standards and environmental samples

2.2.1 | Standards

Ten stock solutions of pure standards (S) containing KNO_3 (N) and NH_4Cl (A), respectively, resulting in five NO_3^- and five NH_4^+ standards, were prepared (codes as follows for NO_3^-: S1N, S2N, S3N, S4N, S5N; and for NH_4^+: S1A, S2A, S3A, S4A, S5A). The standards S1–S3 were prepared to cover the natural abundance range of $\delta^{15}N$ of N_i species (NA standards), ranging from −3.04 to 28.1‰ (Table S2 [supporting information]). Standards S4 and S5 were labeled with ^{15}N (LA) to reach approximately 0.71 and 2.1 at%^{15}N (Table S2 [supporting information]). All LAs were prepared by mixing KNO_3 and NH_4Cl enriched with ^{15}N at 98 at% with the same salts at natural ^{15}N abundance level, respectively, to achieve the isotope ranges. Standards were dissolved in deionized water to achieve a stock concentration of the analyte of 1M in N. Participants were asked to dilute the standards to the concentrations needed by their respective methods and to analyze them in four replicates (n = 4). The "true" $\delta^{15}N$ values of NO_3^- and NH_4^+ salts (chemical purity > 99.9%) were derived from analyzing the standards using EA-IRMS by two to three different laboratories (n = 2–3; see below). Participants were asked to include blanks (e.g., the solvent used to dilute the standards) in the analysis and to report recovery rates of the N species.

In addition to the single compound standards, mixed standards were prepared to test for nonspecificity or possible cross-contamination of the target compound by other N forms (S1Nx, S1Ax). The mixed standards contained the target compound at NA level (here, S1) with the addition of the other N forms (NO_3^-, NH_4^+, amino acid mix) enriched with ^{15}N (at 0.71 at% ^{15}N) (Table S2 [supporting information]). The concentrations of all three N forms were equimolar, that is, at 0.33M in N. All standard solutions were dispatched in 2 ml aliquots.

2.2.2 | Environmental samples

In addition to the standards, five different environmental samples (three soil extracts and two water samples) were collected in March 2012 near Vienna, Austria, to test for consistency of $\delta^{15}N$ values of N_i species in real-world samples across the methods (Table S3 [supporting information]). In short, 3 kg of soil was sampled from surface layers (0–20 cm) in two forests and one grassland ecosystem (P1S, P2S, HWS). Soils were sieved through a 2 mm mesh and extracted with 0.5M K_2SO_4 in the lab (1:5 soil:extractant solution [w:v]). Water samples were collected from two adjacent sites, that is, from a floodplain lake and the Danube River channel (DL, DR). The samples were filtered first with a microcloth filter (Calbiochem) and then through glass fiber filters (Whatman GF/F, cat no 1825-047) applying a gentle vacuum. From each site, four replicates were taken, which were then pooled. The concentrations of NO_3^-, NH_4^+, and DON were determined in all environmental samples by routine procedures as detailed by Hood-Novotny et al (2010) (Figure S3 [supporting information]). Accordingly, the NO_3^- concentrations ranged between 24 and 212 μmol L^{-1} (with one lake water sample having no NO_3^-), the NH_4^+ concentration ranged between 2 and 38 μmol L^{-1}, and DON concentrations ranged between 13 and 142 μmol L^{-1} (with one river water sample having no DON) (Table S3 [supporting information]). The concentrations of nitrite (NO_2^-) were below detection limit in all samples (<0.25 μmol L^{-1}; data not shown);

thus, no measures were taken to remove NO_2^-. To inhibit microbial growth, both soil and water samples were autoclaved with loosened stoppers at 121°C for 1 h. All sample bottles were stored at −20°C until dispatched to the participants. All participants were asked to freeze the environmental samples until analysis and to analyze them in four replicates (n = 4).

2.3 | Nitrogen isotope analysis

In the following, we introduce briefly the different methods and also variations between individual protocols implemented here. We divide them and treat them as different methods (CM—further divided into CM-N_2O and CM-N_2—MD, and DN methods), although we are aware that we are in fact dealing with different method groups and that the application of individual methods can vary quite significantly. Some methods consist also of a combination of methods (e.g., the analysis of NH_4^+ with DN and CM-N_2O includes an MD step followed by alkaline persulfate oxidation, APO), as can be seen in Figure S1 (supporting information).

2.3.1 | CM methods

CM methods transform NO_3^- and NH_4^+ to either N_2O (CM-N_2O) or N_2 (CM-N_2) before isotope analysis. The CM methods applied in this interlaboratory comparison differed significantly between the participating laboratories (Table S4 [supporting information]). Briefly, the CM-N_2O approach utilizes VCl_3 (L1)[16,36] or cadmium (L2, L3)[36,37] to reduce NO_3^- to nitrite (NO_2^-), which is finally converted to N_2O by azide (L1, L2)[16,36] or hydroxylamine (L3).[38,39] If azide is used, one extraneous N is introduced into the analyte, and if the less toxic hydroxylamine is used, the conversion is not quantitative (Table S4 [supporting information]). For isotope analysis of NH_4^+, the N_i species is either oxidized to NO_3^- by persulfate digestion and further reacted to N_2O as described earlier (L1),[16] or sequentially transformed via NO_2^- to N_2O by hypobromite (BrO^-) with and without azide (L2, L3[40,41]). In the absence of azide (L3), N_2O is a side product, and the methods can be applied only to labeled samples (L3). Ammonia is best isolated before the conversion to N_2O, for example, via MD (L3; not done by L2). With the CM-N_2 approaches, NH_4^+ is converted to N_2 by BrO^- after isolating the N species with MgO and acid trap (L4).[42] Nitrate is converted to N_2 by pyrolysis, or through thermal conversion (e.g., TC/EA), after isolation from other N-bearing species by ion exchange and/or precipitation (L4, L5).[26,43] The CM-N_2O approach utilizes continuous flow PT-IRMS techniques, whereas CM-N_2 methods can also employ dual-inlet IRMS systems or TC/EA-IRMS techniques (Table S4 [supporting information]). There are basic differences in sample size requirements, which limit quantification and precision. Sample volumes ranging from 2.5 to 40 mL and 230 to 1000 mL were used for CM-N_2O and CM-N_2, respectively (Table S4 [supporting information]). Generally, CM-N_2O methods are more suitable for low-concentration ^{15}N analysis (in the nmol range). Average optimal N concentrations as used by the laboratories were 0.312 and 150 μmol N for CM-N_2O and CM-N_2, respectively (NA analysis of ^{15}N) (Table 1). In general, about 50 samples can be processed for both NO_3^- and NH_4^+ per week (5 days) with CM-N_2O, and about 30 samples with CM-N_2 (supporting information), though the turnover varies considerably between methods applied.

2.3.2 | MD method

The MD method is based on outgassing dissolved NH_4^+ as NH_3 under alkaline conditions and subsequent conversion to NH_4^+ salt under acidic conditions. MD was introduced decades ago[18–20] and is still one of the most widely used procedures to analyze ^{15}N abundance in N_i. Most laboratories that employed the MD method in this interlaboratory comparison (L6–L9) used one of the original methods, with variable modifications (Table S5 [supporting information]).[16,19] In brief, 10–100 mL of sample was diffused for 3–5 days (L6–L8) or 20 days (L9) after the addition of MgO (L6: 100 mg; L7–L8: 200 mg; L9: 600 mg), and NH_3 was trapped on acid traps (disks of filter paper or glass fiber filter amended with $KHSO_4$, H_2SO_4, or oxalic acid) wrapped in semipermeable Teflon membrane floating on the solution. For placement within the sample, disks can also be generally fixed to stainless steel wires as, for example, in one of the original methods.[18] After NH_4^+ was quantitatively removed by MD, NO_3^- was analyzed by the same method after reduction of NO_3^- to NH_4^+ with Devarda's alloy (L6: 50 mg; 400 mg: L7–L9).[21] MD samples are analyzed by continuous flow EA-IRMS, that is, combustion without using PT technology, and therefore, the MD method requires a relatively higher sample amount (in the μmol range). The average optimal N concentration as used by the laboratories was 7 μmol N for MD (Table 1). The throughput is dependent on the incubation time, and 70 and 100 samples can be processed for $\delta^{15}N$ analysis of NH_4^+ and NO_3^- isotope analysis, respectively, within a week (with a maximum of 100 and 150 samples possible with 3 days' incubation time).

2.3.3 | Biological conversion by the DN method

In the DN method, NO_3^- is selectively converted to N_2O by denitrifying bacterial strains of *Pseudomonas aureofaciens* or *Pseudomonas chlororaphis*, both of which are lacking nosZ, the gene coding for N_2O reductase, which would further reduce N_2O to N_2.[27,28] Denitrifying enzymes have high substrate specificity and affinities (K_m values) in the nM range; thus, NO_3^- is quantitatively converted to N_2O, which can then be analyzed using PT-IRMS. Laboratories L10, L11, and L13 used a *P. aureofaciens* strain, and L12 used a *P. chlororaphis* strain for this reaction (Table S6 [supporting information]). All laboratories followed the original protocol by Sigman et al,[1] where the method was first presented, with small adjustments, except laboratory L11, which had adopted

TABLE 1 Overview of characteristics, requirements, and basic performance of the various methods used in this interlaboratory comparison to analyze $\delta^{15}N$ signatures of inorganic Nitrogen (N) forms

Method (abbreviation)	Sample volume (mL)	Optimum N concentration (μM)	Optimum amount of N (μmol)	Recovery (%)[a]	Recovery (%)[b]	Blank (%)	Blank (μM)	Blank (μmol)	Participating laboratories
Chemical method—N_2O (CM-N_2O)	9.25 (40 for L3)	32 (300 for L3)	0.312 (12 for L3)	97.0 ± 4.51 (25% for L3)	103 ± 6.0 (n.d. by L3)	9.20 (0.00 for L3)	2.94 (0.00 for L3)	0.029 (0.00 for L3)	3
Chemical method—N_2 (CM-N_2)	1810	83	150	Not reported	Not reported	0.00	0.00	0.00	2
Microdiffusion (MD)	42	170	7.00	88.2 ± 3.05	115 ± 8.5	4.30	7.31	0.301	4
Denitrification (DN)	2	50	0.100	101 ± 8.1	106 ± 8.6	4.1	2.05	0.004	4

Note: The average of all reported values is presented method-wise. L3 used a conventional CM-N_2O method where N_2O is a by-product of the reaction between hydroxylamine and NO_2^- and is thus presented separately.

[a]As determined from single standards at natural abundance (S1–S3).

[b]As determined from mixed standards (S1Nx, S1Ax) where target compound was at natural abundance and nontarget compounds were labeled. The average recovery is shown.

the protocol of Morkved et al[44] for *P. aureofaciens* (more details in supporting information). Ammonium was measured by the DN approach after MD of NH_4^+ and persulfate digestion, which oxidizes all NH_4^+ to NO_3^- (L13). The optimum amount of N used by the laboratories was with an average of 0.1 μmol N, lowest for DN. The throughput is similar to CM-N_2O: about 45 samples can be processed per week for $\delta^{15}N$ analysis of NO_3^-, and 4 days at a minimum in the case of NH_4^+ analysis by MD and APO preprocessing. However, bacterial cultures need to be prepared and maintained, which makes this method more labor-intensive compared to the other tested ones.

2.3.4 | Freeze-drying and evaporation followed by EA-IRMS analysis

The relatively simple freeze-drying and evaporation technique (EA) was carried out by L14, L15, and L16. With this technique, an appropriate amount of the standard was freeze-dried, resuspended with distilled water (various amounts; 50–170 μL), pipetted into tin cups, and, subsequently, placed in an oven at 50°C or under a fume hood at room temperature until dry. The resulting NO_3^- and NH_4^+ salts were analyzed using EA-IRMS. This technique is suitable for pure dissolved target N species only, as it was the case for standards S1–S5 and cannot be applied to natural environmental samples.

2.4 | Data processing and statistics

We calculated $\Delta\delta^{15}N$ values as the difference between raw $\delta^{15}N$ values obtained by each laboratory and the reference ("true") value obtained by the EA method for each standard. The EA results served as a reference in this study, as freeze-drying and/or evaporation followed by EA-IRMS are the most conventional and direct methods of isotope analysis (without any isolation steps). It should be noted that this EA approach does not provide a certified reference value, but one that is sufficiently accurate here to compare the relative precision and accuracy of the other methods. More details on how the reference value was established are given in the supporting information. Accuracy is given as the difference of $\delta^{15}N$ values of NA standards from true $\delta^{15}N$ values as $\Delta\delta^{15}N$ and as $\Delta at\%^{15}N$ for LA standards. To better represent the variability in the data set, we report results as mean with SD as well as median values for each method, with upper and lower quartiles (interquartile range: 25th to 75th percentiles expressing reproducibility) in Figure 1. Precision is given as the mean SD (± relative SD) of results received from each laboratory and expresses thus within-laboratory or intralaboratory precision. Interlaboratory precision equals the SD across the means of each method group.

Nonspecificity of each method was calculated using S1Nx and S1Ax results, assuming that a complete conversion of both nontarget N species would result in a 100% contamination, and was computed using the following equation:

$$\text{Nonspecificity} = \frac{at\%^{15}N_m - at\%^{15}N_T}{(f_T * at\%^{15}N_T + f_{NT1} * at\%^{15}N_{NT1} + f_{NT2} * at\%^{15}N_{NT2}) - at\%^{15}N_T}$$

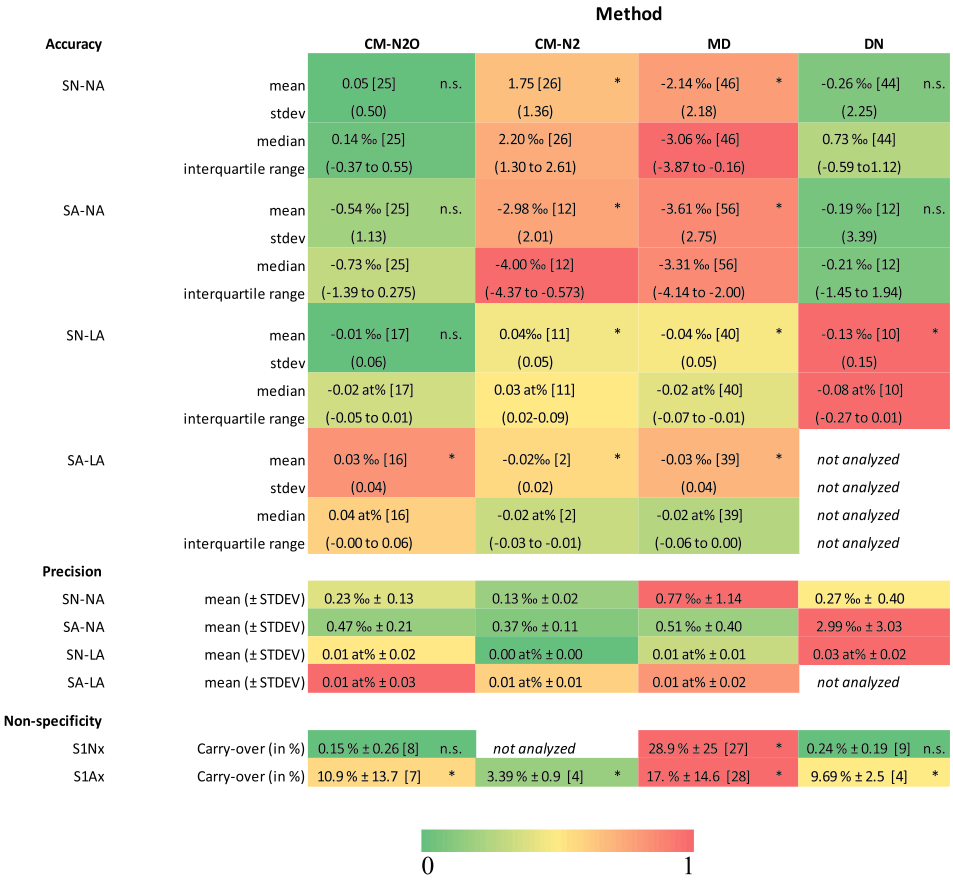


FIGURE 1 A heatmap illustrating the accuracy, precision, and specificity of the methods used by variable laboratories in this interlaboratory comparison to analyze $\delta^{15}N$ in inorganic nitrogen (N) forms. Methods tested were the chemical method transforming inorganic N forms to N_2O (CM-N_2O), the chemical method transforming inorganic N forms to N_2 (CM-N_2), the microdiffusion (MD) method, and the bacterial denitrifier (DN) method. Results are presented for natural abundance standards of NO_3^- (NA-SN) and NH_4^+ (NA-SA) and for labeled standards of NO_3^- (LA-SN) and NH_4^+ (LA-SA). Color codes were normalized to the maximum value found for each category, where the dark green color (closer to 0) indicates highest accuracy, precision, and lowest nonspecificity (demonstrating better performance of the method utilized by one lab) and the red color (closer to 1) indicates lowest accuracy, precision, and lowest nonspecificity. Yellow colors indicate intermediate values. Accuracy is given as the difference of $\delta^{15}N$ value of NA standard from true $\delta^{15}N$ value as $\Delta\delta^{15}N$ [n] and as at% offset for LA standards. Data are mean and median values and standard deviation (SD) including lower (25%) and upper (75%) quartile ranges. Precision is given as the mean SD (± relative SD; in ‰ for NA and in at% ^{15}N for LA) of results received from each laboratory (intralaboratory precision). Nonspecificity was calculated assuming that a conversion of nontarget N species would result in 100% contamination (more details in the main text). The $\Delta\delta^{15}N$ results of S1, S2, and S3 (NA) and S4 and S5 (LA), which were characterized by different isotope signatures (NA) or isotopic enrichment (LA), respectively, were pooled. Asterisks indicate significant differences at $P < 0.05$ [Color figure can be viewed at wileyonlinelibrary.com]

where at%^{15}N is atom-percent ^{15}N, m = measured, T = target compound (NA values of NO_3^- and NH_4^+ in S1Nx and S1Ax, respectively), NT = nontarget compounds (0.71 at% ^{15}N; combined with other N containing sources), and f represents the added fractions of T and NT (1/3 each).

All statistical analyses were carried out using the IBM SPSS 25 statistical software. For standards, significance of differences between individual lab results and results grouped into the different methods, respectively, and the "true" values based on the EA method were tested using two-sample Student's *t*-tests (independent sample's *t*-test). Differences were assumed significant when $P < 0.05$. For environmental samples, all individual lab results and means of each method were compared with the mean value calculated from the results of all methods used. Deviations from this mean were evaluated using Student's *t*-tests as mentioned earlier. Before the use of any statistical method, the data were checked for normality (Kolmogorov–Smirnov's test) and for homogeneity of variances (Levene's test). Further details on statistical analysis are provided in the supporting information.

However, it must be noted that this interlaboratory comparison was not designed as a proficiency test, because participants were not judged for the acceptability and traceability of their results, and there was no reference laboratory or standard operating procedures recommended. Outliers (intralaboratory extreme values) were, however, identified based on reported laboratory batch means.[45] Participants were asked to perform their routine analysis. Nevertheless, the procedures used by each laboratory were considered appropriate to the objective of the study. Rather, the purpose of the interlaboratory

comparison was to determine whether the most commonly used methods to analyze $\delta^{15}N$ in N_i produce acceptable, concordant, and intercomparable results when performed by different laboratories.. As a result, the study has a more descriptive character, and we report and discuss discrepancies in the data from each group of methods rather than following strict metrological protocols.

3 | RESULTS AND DISCUSSION

3.1 | Natural ^{15}N abundance standards

In the absence of other confounding N_i or organic N species, repeated analysis of NO_3^- and NH_4^+ salts yielded information about the accuracy and precision of isotope ratio measurements for each method while, at the same time, allowing evaluation of the performance of each participating laboratory. For NA nitrate standards (SN-NA), the mean (±SD) and median $\Delta\delta^{15}N$ values reported from laboratories using the CM-N_2O technique were +0.055 ± 0.50‰ and +0.138‰, respectively, and the results did not differ significantly from the reference method ($P = 0.501$) (Figure 1; Figure S1 [supporting information]), with no significant differences found between SN1, SN2, and SN3 (Figure S2 [supporting information]). Each individual SN-NA standard measured by the contributing laboratories applying the CM-N_2O method in quadruplicates also exhibited smallest deviations in the actual $\delta^{15}N$ value (always less than 1‰), as compared to the other methods (Figure S4A [supporting information]). Regarding precision, laboratories using the CM-N_2O method reported with 0.229‰ overall intermediate intralaboratory precision for SN-NA (Figure 1; Figure S5 [supporting information]). Laboratories using MD reported the lowest overall precision (Figure 1; Figure S5 [supporting information]) and the lowest accuracy at natural abundance levels; the mean $\Delta\delta^{15}N$ of −2.14 ± 2.18‰ (median −3.06‰) was significantly different from the reference method ($P = 0.001$). The reported accuracy from laboratories applying both DN and CM-N_2 laid between that of CM-N_2O and MD for SN-NA, with mean $\Delta\delta^{15}N$ values of −0.26 ± 2.25‰ for DN and +1.75 ± 1.36‰ for CM-N_2 (median: +0.73‰ and +2.20‰, respectively). The mean $\Delta\delta^{15}N$ value was statistically different for laboratories applying CM-N_2 methods compared to the reference method ($P = 0.001$) but not for those applying DN ($P = 0.477$); however, variability for DN was high (Figure 1; Figure S1 [supporting information]). Overall precision was highest for laboratories using CM-N_2 and intermediate for those using DN when analyzing SN-NA (Figure 1; Figure S5 [supporting information]). The precision was for all methods (on average 0.7‰) within the lower range of published values[16] but considerably higher (about 3 times) than interlaboratory precision (Figure 1; on average 1.94‰), indicating that the spread in mean values was largely due to differences in results reported from different laboratories (which was the case for all results reported; see below).

Analysis of NH_4^+ standards at NA (SA-NA) gave similar results, though differences to the actual values were on average larger (Figure 1; Figures S2–S4 [supporting information]), indicating lower accuracy of SA than SN methods with the techniques compared here from variable labs. The accuracy was best among laboratories applying the CM-N_2O methods, followed by those applying DN, and least accurate results were obtained by the laboratories applying MD and CM-N_2 methods (Figure 1; Figures S2 and S3 [supporting information]). Similar to SN-NA, the results reported from laboratories using the CM-N_2O and DN methods were on average not statistically different from the actual $\delta^{15}N$ value for SA-NA ($P = 0.136$ and 0.904, respectively) (Figure 1; Figure S1 [supporting information]). Overall precision was not as good for SA-NA as for SN-NA, with laboratories using DN methods showing lower precision than other methods (Figure 1; Figure S5 [supporting information]); however, only one laboratory employed the DN method for NH_4^+ and used MD in combination with persulfate digestion to collect and oxidize NH_4^+ to NO_3^- before DN measurements. Thus, no general conclusion can be drawn on the accuracy and precision of DN for $\delta^{15}N$ analysis of NH_4^+ here.

Laboratories reported recovery rates of >95% (Table 1) for CM-N_2O for both SN and SA, and thus, isotope fractionation during sample preparation was of minor importance,[15] most likely explaining the high accuracy of CM-N_2O methods for pure standards.[16,37,46] Blanks were significant with the CM-N_2O methods employed by the participating laboratories (on average 9.20% of sample peak or 0.03 μmol for both NO_3^- and NH_4^+ analysis in this ring test; Table 1), though they were higher here than reported elsewhere for the same methods.[16,36,37,47] However, it seems that proper blank correction could be achieved. The high blank could explain the intermediate precision found for this method by the participating laboratories.

The laboratories utilizing the DN methods also yielded satisfactory results analyzing standards with acceptable intralaboratory precision (at least for SN-NA), though the variability of $\Delta\delta^{15}N$ values was relatively high for this method, with both over- and underestimation of the actual $\delta^{15}N$ value (Figure S4A [supporting information]). Underestimation of $\delta^{15}N$ values can result from isotopic fractionation associated with incomplete conversion.[33,48] Recovery rates were quite variable, but on average close to 100% (101% ± 8.1%; Table 1), and not related to the $\delta^{15}N$ value deviation found (data not shown), suggesting that isotope fractionation was of minor importance. Previous studies have shown that the conversion of NO_3^- to N_2O by DN is quantitative and complete under optimal conditions (e.g., Jantti et al[33] and Casciotti et al[48]) and that results are generally robust.[25,28,44,49,50] Under- and overestimation of isotope values by DN methods can further result from isotope normalization or blank issues. Because all laboratories applying DN methods also utilized certified standards, not the reference N_2O tank, as a means of calibration of isotope results, along with the samples (similar to CM-N_2O above), normalization issues are less likely. The systematic shift in $\Delta\delta^{15}N$ between S1, S2, and S3, from overestimation in S1 and S2 to underestimation in S3 (Figures S1 and S2 [supporting information]; at least for NO_3^- analysis; NH_4^+ results cannot be properly evaluated due to the scarce dataset), suggests a two-source mixing behavior and a significant impact of the blank. DN methods have manifold blank

issues, including residual NO_3^- in the culture medium, extraneous NO_3^- used during preculturing not being removed completely, N_2O from denitrification of extraneous NO_3^- dissolved in the medium or in the bacteria not being removed completely by Helium sparging, and contamination with atmospheric N_2O.[28,33,50] Blanks were relatively low from the reported values for DN (on average 4.1% of the sample peak, i.e., 4 nmol in this ring test; Table 1). Nevertheless, it seems that proper blank correction would be necessary. Most laboratories utilizing DN, including the ones participating here, conduct the blank correction together with the calibration of the samples utilizing in-house nitrate reference standards. This is not the most accurate way for a blank correction, because first, the sample-to-blank ratio needs to be kept constant, and second, the blank would need to be precisely quantified for the whole DN procedure.[51] One laboratory also reported contamination problems with some DN analysis (data not shown). It has to be kept in mind that the number of labs employing DN was relatively small in this study.

Although laboratories applying the CM-N_2O and DN results reported on average not significantly different results to the actual $\delta^{15}N$ values, laboratories applying MD underestimated the actual $\delta^{15}N$ value significantly for both SN and SA by several ‰ (Figure 1; Figure S1 [supporting information]). This is most likely due to the relatively low recovery for this method (88.2% ± 3.05) and the associated isotope fractionation effects with incomplete recovery.[15,52] MD of NH_3 by volatilization is a slow process that is susceptible to strong isotopic effects (20–30‰[53]). Indeed, issues with low recoveries for MD have been reported previously,[20,33,54] especially if the MD fails (e.g., acid traps sticking to the container walls,[19] incubation bottles or acid traps leaking,[20] inappropriate headspace/sample ratio[52]), which causes isotope fractionation resulting in more negative $\delta^{15}N$ values.[15] For MD, it is important to include standards to correct for isotope fractionation due to incomplete N_i recovery.

The laboratories using the CM-N_2 methods over- and underestimated the $\delta^{15}N$ values for NO_3^- and NH_4^+, respectively. For NO_3^-, both laboratories used the ion exchange method to collect NO_3^-, a technique prone to preferential retention/elution of $^{15}NO_3$,[26] likely explaining the overestimation of the $\delta^{15}N$ values (though this was true only for L5). For NH_4^+, the traditional steam distillation method was used by L4 before oxidation to N_2 by LiOBr; the method also includes an acid trapping step of NH_4^+. Thus, there is a potential bias due to isotope fractionation or incomplete recovery, NH_3 distillation, or loss of N during collection,[55] likely causing the underestimation of the $\delta^{15}N$ values. Generally, the low number of participating laboratories makes it difficult to evaluate CM-N_2 methods as one group.

3.2 | Labeled standards

Similar to NA standards, laboratories using the CM-N_2O methods reported the most accurate results when measuring the LA, but only for the SN-LA standards, where the only nonsignificant difference to the actual value was found ($P = 0.605$) (mean $\Delta at\%^{15}N = -0.012\%$, median $\Delta at\%^{15}N = -0.019\%$). The $\Delta at\%^{15}N$ values were significantly different from those of laboratories using MD ($P = 0.001$) (mean $\Delta at\%^{15}N = -0.042\%$, median $\Delta at\%^{15}N = -0.021\%$), CM-$N_2$ ($P = 0.002$) (mean $\Delta at\%^{15}N = +0.041\%$, median $\Delta at\%^{15}N = +0.025\%$). and DN ($P = 0.002$) (mean $\Delta at\%^{15}N = -0.131\%$, median $\Delta at\%^{15}N = -0.073\%$). For individual lab results of each SN-LA standard, most results were statistically different from the actual value, with the exception of two laboratories using MD (25% of the labs participating with MD) (Figure S4B [supporting information]). Intralaboratory precision was best for laboratories applying CM-N_2, followed by MD, CM-N_2O, and DN (Figure S5 [supporting information]). When SA-LA standards were analyzed, all laboratories yielded $at\%^{15}N$ values, which were significantly different from the actual value with their methods used (Figure 1; Figures S1–S3 and S4B [supporting information]), but the mean differences were smallest for CM-N_2 (mean $\Delta at\%^{15}N = -0.018\%$, median $\Delta at\%^{15}N = -0.018$ $at\%^{15}N$), followed by MD (mean $\Delta at\%^{15}N = -0.026\%$, median $\Delta at\%^{15}N = 0.016$ $at\%^{15}N$) and CM-N_2O (mean $\Delta at\%^{15}N = +0.033\%$, median $\Delta at\%^{15}N = +0.035$ $at\%^{15}N$). Contrary to NA standards, laboratories using MD methods yielded one of the best results at least for SA-LA.

As fractionation effects can be largely neglected in labeled samples, MD methods, which frequently show recovery issues, performed better with LA as compared to NA standards in this interlaboratory comparison. The insensitivity of ^{15}N analysis to recovery in MD with ^{15}N-enriched samples was also demonstrated by others.[20] An underestimation of the actual $\delta^{15}N$ value as found with most methods can be explained by the fact that IRMS is not designed to measure relatively highly labeled samples, as isotope fractionation can occur in the IRMS that does not scale linearly with isotopic composition (so-called scale compression/expansion).[56] An overestimation of isotope values in ^{15}N-labeled samples, as observed here from laboratories using CM-N_2O with SA-LA standards and with CM-N_2 (L5) with SN-LA, can only be explained by problems with blank correction, with isotope calibration (CM-N_2O),[20,26] or with memory effects.[57] It has to be noted that though the offsets of the LA standards were large, they are of less concern in ^{15}N tracer studies, where NO_3^- and NH_4^+ are enriched at several at% ^{15}N. Here, the isotopic offsets in the at% scale ranged between 2 and 6% relative error at 0.7 $at\%^{15}N$ and between <1 and 3% relative error at 2.1 at $\%^{15}N$ and were thus in an acceptable range for all methods employed.

3.3 | Mixed standards and nonspecificity

For S1Nx, laboratories applying the CM-N_2O and DN methods showed no carryover and reported accurate results for the target compound (Figure 1). Production of N_2O from NO_3^- by denitrifiers is highly specific and does not pose risks for cross-contamination.[25,28,33,50] Our comparison demonstrates that also the CM-N_2O methods based on the reduction of NO_3^- by VCl_3 or Cd via NO_2^- to N_2O are unbiased by interference with organic and

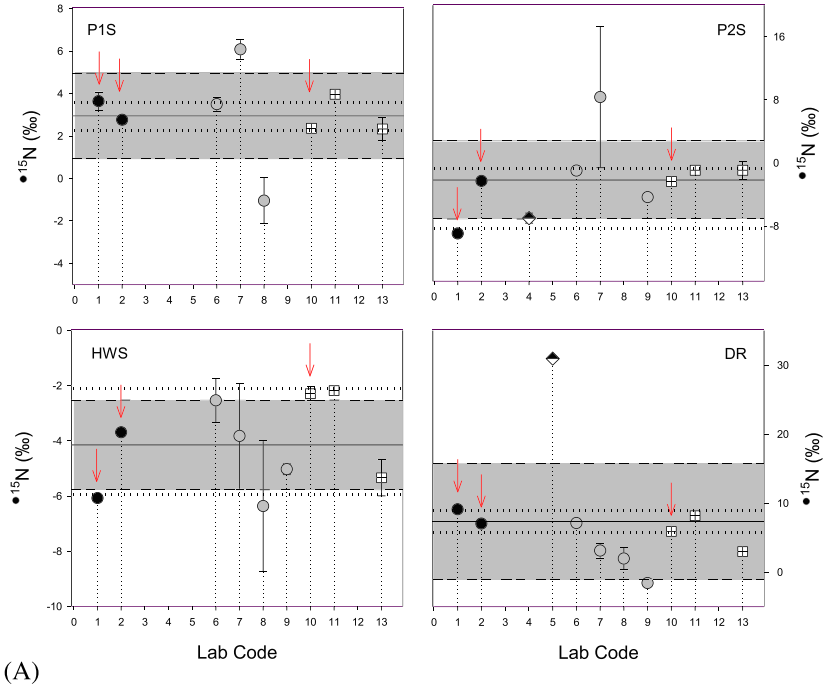
other N_i compounds. Only laboratories using MD showed significant cross-contamination and yielded, thus, unsatisfactory results when analyzing S1Nx standards (Figure 1). Three out of four labs obtained results, which were significantly enriched in ^{15}N and were thus impacted by N from nontarget compounds (Figure S6 [supporting information]). Average nonspecificity was 28.9 ± 24%, with up to 52% of nontarget compounds being carried over (L9). Thus, a variable but potentially large amount (up to half) of non-NO_3^-, that is, NH_4^+ or amino acid-N, ended up in the acid traps during MD.

Laboratories applying MD also showed issues with nonspecificity for S1Ax, though the carryover was lower (on average 18.0 ± 15%) compared to S1Nx (Figure 1; Figure S6 [supporting information]). The contamination is of different nature for NH_4^+ as compared to NO_3^-, because both AA and NH_4^+ can end up in the "NO_3^- pool" after being reduced to NH_4^+, whereas it is likely that only AA end up in the NH_4^+ pool, causing lower carryover issues. This is because NO_3^- is relatively stable in solution, whereas AA is easily broken down.[58] Generally, the high nonspecificity was corroborated by higher yields of S1x standards analyzed by MD as opposed to CM-N_2O and DN (Table 1). The reasons for high nonspecificity by MD can be manifold. First, the addition of MgO to raise the pH to >10 can cause carryover due to alkaline hydrolysis of DON and deamination of amino acids and thereby contamination of the NH_4^+.[15,20,33,41,59] Second, the Devarda reaction can cause reductive breakdown of organics or other O-containing substances.[35,41] This is likely due to the harsh conditions generated by the Devarda's alloy and the high pH. In addition, NH_4^+ needs to be properly removed before isotope analysis of NO_3^- via MD.[20] Using an optimized method to isolate NH_4^+ by MD resulted in very low carryover by L1, a lab that otherwise used CM-N_2O to further process the trapped NH_4^+ by alkaline persulfate oxidation. Correlation analysis did not indicate a significant relationship between the magnitude of nonspecificity and any methodological factor, though we noticed that higher amounts of MgO and longer incubation times resulted in increased carryover, as suggested also by others (e.g., Ros et al[35]). It has to be noted that carryover due to breakdown of DON or NH_4^+ carryover by MD is an issue for both NA and LA samples. In a method study by Jantti et al[33] where labeled samples were investigated to test proper methods for studying the ^{15}N isotope pool dilution method to measure gross nitrification rates, MD produced significantly lower at%^{15}N values of NO_3 as compared to DN. The underestimation of at%^{15}N values resulted in a significant (up to 10-fold) overestimation of gross nitrification rates by MD.

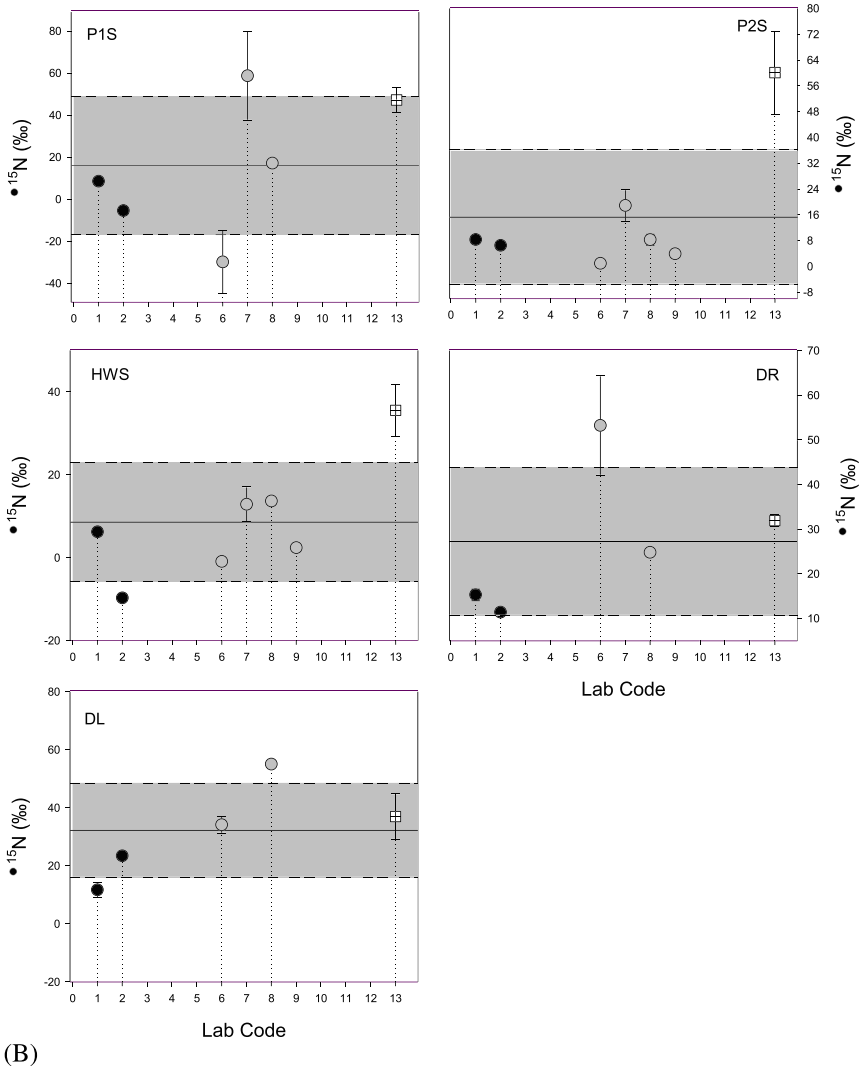
In addition to MD, also one out of two laboratories using CM-N_2O methods showed high nonspecificity for S1Ax (L2; 21%) (Figure S6 [supporting information]). This method is based on the reaction of NH_4^+ with hypobromite to form NO_2^- and later N_2O and does not—contrary to the method used by L1—isolate NH_4^+ before conversion to N_2O. Other organic compounds broken down by this reaction raise concerns on the use of this method,[46] an issue that could be solved by pre-isolating NH_4^+ before oxidation.[22] L13 measured $\delta^{15}N$ in NH_4^+ by DN after isolating NH_4^+ by MD, followed by persulfate oxidation to NO_3^-, a method that yielded significant carryover by the laboratories using it (9.7%; Figure S6 [supporting information]), likely due to issues with MD. Similarly, carryover was found to be significant for L4 (3.4%), using the steam distillation method after isolating NH_4^+ by acid traps before oxidation to N_2 with LiOBr. This method is afflicted with a high risk of nonspecificity, due to the aforementioned problems with acid traps, high distillation temperatures, and subsequent decomposition of organic N.[41]

3.4 | Environmental samples

Although the analysis of pure NO_3^- or NH_4^+ salts produced acceptable results for most methods applied by the participating laboratories, more divergent $\delta^{15}N$ values were reported when the isotopic composition of NO_3^- and NH_4^+ in the environmental samples was analyzed (Figure 2). Concentrations of NO_3^- and NH_4^+ were low to intermediate, ranging from 24 to 212 μmol l^{-1} and from 2 to 38 μmol l^{-1}, respectively, but within the range typically found for water samples and soil extracts (Table S3 [supporting information]). Concentrations of DON were from less than 10 μmol l^{-1} up to 142 μmol l^{-1} also in the low to high range (Table S3 [supporting information]). Because the actual $\delta^{15}N$ values from the environmental samples were not known, we calculated the mean deviation and SD across all results. SD across all laboratories and methods ($n = 8–10$ for each sample) reported for the environmental samples was 2.01‰ for PS1, 4.86‰ for PS2, 1.62‰ for HWS, and 8.43‰ for DR for NO_3^- analysis, with all laboratories and methods showing a similarly high variability. The maximum difference between two reported values was as high as 32.5‰ for DR. If SDs were calculated only for laboratories L1, L2, and L10 (laboratories that provided accurate and unbiased results for the standards; Figure 1), the SDs were on average smaller but still substantial for NO_3^- (0.65‰ for PS1, 3.83‰ for PS2, 1.91‰ for HWS, and 1.62‰ for DR), rendering high uncertainties in the reported mean values. SD across laboratories was even higher for NH_4^+ isotope results in the environmental samples ($n = 5–7$): 32.9‰ for PS1, 20.5‰ for PS2, 14.4‰ for HWS, 16.6‰ for DR, and 16.2‰ for DL. SDs within laboratories were for both NO_3^- and NH_4^+ found to be considerably lower (on average 3.1‰), indicating again that the spread in mean values was largely due to differences in results reported from different laboratories. Given the high deviations between reported values for standards, we could not narrow down the "real" isotope value of NH_4^+ for the environmental samples based on the interlaboratory comparison. Although interlaboratory variability was very large and not connected to any methodological or sample factor (e.g., DON content, content of target compound), the intralaboratory variability or precision of $\delta^{15}N$ of environmental samples correlated negatively with the target concentration for NO_3^- analysis but not for NH_4^+ analysis (Figure S7 [supporting information]). The unsatisfying performance of each method or laboratory for environmental samples is, for one, connected to the shortcomings of each method (as discussed earlier) and related



(A)



(B)

FIGURE 2 Legend on next page.

FIGURE 2 The $\delta^{15}N$ results from isotopic analysis of environmental samples by different laboratories (A, $\delta^{15}N$ of NO_3^-; B, $\delta^{15}N$ of NH_4^+). The solid line indicates the average of all the results, plus minus standard deviation (±SD; shaded area). Red arrows indicate results from laboratories showing high precision and selectivity when analyzing standards, and the dotted line indicates the SD when calculating the average from those results. P1S, P2S, and HWS are soil extracts, and DR and DL are river samples (for more information, refer to the text and Table S3 [supporting information]). Black circles (L1–L3) = results from CM-N_2O; semi-filled diamonds (L4–L5) = results from CM-N_2; gray circles (L6–L9) = results from MD; crossed squares (L10–L13) = results from DN. For abbreviations of the methods, see the legend of Figure 1. Note that not all laboratories participating in this intercomparison analyzed ^{15}N from environmental samples (but only from standards, e.g., L3, L12) [Color figure can be viewed at wileyonlinelibrary.com]

to issues with precision, accuracy, blank contamination, and nonspecificity. However, as the laboratories that did not report such problems yielded highly variable results, additional factors like matrix effects (e.g., high salt concentration in soil extracts or marine samples) or the impact of third compounds must be considered.[20,26,60,61] Nitrite was not a problem here, because NO_2^- concentrations were below detection limit (<0.25 μmol l^{-1}) in all environmental samples. However, if NO_2^- is detectable in the samples, it needs to be removed by sulfamic acid or ascorbic acid pretreatment.[51,62,63] However, high salt concentrations should not be a problem for the N_2O-based methods, as supported by published data sets on reference materials prepared in seawater and freshwater (e.g., references 28 and 51) but it can be a problem for DN and CM-N_2.[64,65]

Other method comparisons for $\delta^{15}N$ of NO_3^- in environmental samples showed much better (though also not optimal) agreement between different methods tested, for example, groundwater and surface water samples differed only by 0.4–0.5‰ between MD and CM-N_2 in a study carried out by Sebilo et al.[66] Thus, it shows that high accuracy can be achieved with MD if sufficient care is taken to optimize the MD procedure (Figure 3). Similarly, there was sufficient agreement ($\Delta\delta^{15}N$ of 1.5‰) between $\delta^{15}N$ of NO_3^- of surface waters measured by a CM-N_2 ($AgNO_3$) method and DN, though much larger differences were found for specific samples.[65] Moreover, $\delta^{15}N$ results of NH_4^+ from environmental samples (lake water) measured with the BrO^- oxidation method also agreed sufficiently well with the MD method.[46] However, in all cases, the analyses were always carried out by one laboratory, suggesting that imprecise standardization procedures and erogenous application of methods by some laboratories are eventually responsible for the large spread in the mean values in our case. In agreement with that, Aly et al[67] demonstrated high variability of $\delta^{15}N$ of NO_3^- (up to appr. 5‰) by conducting an interlaboratory comparison of various CM-N_2 and DN methods on standards.

Thus, the variability found here can also result from poor performance and/or weaknesses in one or another laboratory protocol, which were often modified from the original protocol by participants. Though it can be assumed that the participating laboratories are well experienced, the "schooling" and level of training obviously vary. The cause of the problem can also be found in the performance of the instrumentation, laboratory conditions, and other factors, which are difficult to trace back but which contribute to a loss in analytical accuracy and precision achieved by any method in any laboratory. Importantly, however, the high variability between the methods is also due to the unsuitability of specific methods for a particular sample type. For example, samples that contain low amounts of N are not suitable for MD, and so are samples with high amounts of DON. The limit of DON concentration acceptable for analysis with MD is 10 μmol l^{-1},[33] above which DON needs to be removed before analysis of $\delta^{15}N$ of N_i, for example, by microdialysis or using a sodium-acetone mixture to precipitate DON.[68] In case of too low amounts of N, there is the possibility of spiking with known quantities and isotope compositions of N_i, but this works only with labeled samples.[69] The pyrolysis-based $AgNO_3$ method (CM-N_2) is also not suitable for samples with high salt content, and there are also limitations at high salt content with DN. Still these methods are frequently used for such samples and work in soil extracts up to 2 M KCl.[44,70] In Figure 3, we first provide guidelines that should aid in the decision-making process to find the best suitable method for specific sample types, variables, and parameters to optimize the protocols (best-practice guidelines) and further caution notes, to aid in producing the most reliable results. Assuming that our test is representative of laboratories routinely carrying out $\delta^{15}N$ analysis of N_i, our study shows that the current reproducibility is not sufficient for meta-analysis of $\delta^{15}N$ of N_i. We call for improved standardization and better validation of methods used by laboratories routinely conducting $\delta^{15}N$ analysis of N_i, and for more harmonized analytical protocols.

3.5 | Method comparison and recommendations on method improvements

Overall, for standards (both in single form and mixed), laboratories utilizing the CM-N_2O methods performed best in this interlaboratory comparison in terms of precision, accuracy, and specificity, followed by laboratories utilizing DN. CM-N_2O and DN methods have also been previously reported to be accurate and quantitative.[16,36,37,46,47,71,72] Both methods convert N_i forms to N_2O and analyze it using PT-IRMS, thereby pre-concentrating N_2O from a larger gas volume (10–30 mL) before IRMS analysis, and are thus highly sensitive, being able to analyze N_i at low concentrations (<30 nmol).[16,33,71] One reason for the high accuracy is the complete conversion of CM methods, as also confirmed by our study. Another reason may be associated with the fact that certified standards are added through the entire run, which increases sample analysis time but simultaneously eliminates IRMS calibration issues because the standards, and not the N_2O reference tanks, are used for normalization of stable isotope signals. The main disadvantages of the

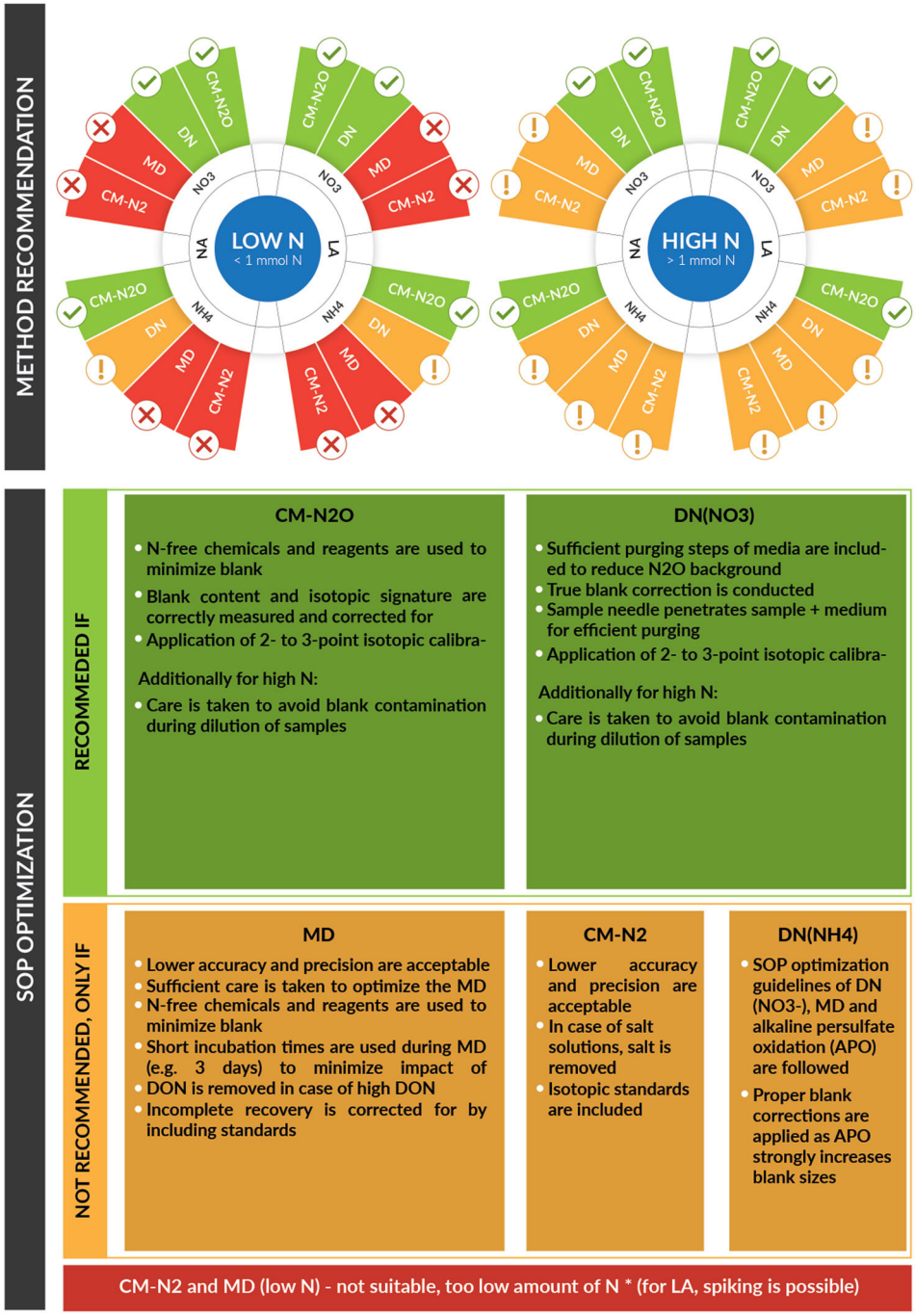


FIGURE 3 Method recommendation for samples with high and low nitrogen content and for natural abundance (NA) and labeled samples for analysis of $\delta^{15}N$ of inorganic nitrogen forms (NO_3^-, NH_4^+). Best-practice guidelines (standard operating procedures and optimization procedures) are included. Note that all methods require proper calibration and normalization procedures. Note also that for labeled samples, it is recommended to use labeled standards and to include "dummies" to avoid memory effects. The check mark and green color mean "recommended," the cross and red color mean "not suitable," and the exclamation mark and yellowish color mean "not recommended, only if." For more details, read the information in the boxes [Color figure can be viewed at wileyonlinelibrary.com]

CM-N_2O methods are the safety precautions required for handling the highly toxic azide reagent and its subsequent disposal (Table S7 [supporting information]). New methods are currently being developed, which hold promise to achieve similar precision and accuracy while utilizing less toxic reagents.[22] Moreover, more suitable N_2O reference materials are currently being developed[73] to implement the recommended two- or three-point calibration approach for CM-N_2O and DN methods also in the future. For CM-N_2O, reliable results can be achieved, even though the blanks are relatively high, and the methods require isotopic reference standards to account for the offset of $\delta^{15}N$ deriving from the incorporation of one N atom in N_2O from azide,[16,36,37,47] which involves extensive correction of the raw results in CM-N_2O methods. Some blank issues were noted from laboratories using DN, though the blank was very low, likely due to improper blank correction; direct measurements of the blank or employing indirect calculation methods/regression equations to account for $\delta^{15}N$ and the size of blanks is generally recommended for all methods, even when blanks are low.[25,28,44] For DN, further improvements such as the one recently published by Zhu et al,[50] which involves the removal of background NO_3^- in the media by anoxic preincubation with *Pseudomonas denitrificans*, need to be achieved to reduce the blank size and to increase the accuracy of

$\delta^{15}N$ analysis of NO_3^-.[51] The main weakness of DN is, however, the requirement and maintenance of special denitrifier cultures, which may be impractical for some laboratories (Table S7 [supporting information]).

Laboratories applying the MD methods, on the contrary, performed inferior for NA standards, both in single and in mixed forms. The main problem with this method is the low recovery, on the one side, and the high risk for carryover, on the other side (Table S7 [supporting information]). Improvements may be achieved by using NaOH instead of MgO, by keeping the incubation times short, by adopting cleaning techniques to properly remove NH_4^+ before analysis of NO_3^-, by avoiding oversaturation of the acid traps, by using vessels with high surface–headspace ratios during MD, by using high-purity KCl and Devarda's alloy, and/or by removing DON before the analysis of N_i.[20,21,35,52,74,75] Measurements of blanks and of standards within appropriate concentration ranges of the samples by MD are highly recommended to correct for the blank contribution and isotope fractionation due to incomplete recoveries in NA studies. Another disadvantage of the MD approach is the relatively large sample requirement and the high N concentrations/amounts needed (> 1 μmol N). CM-N_2 methods have similar disadvantages but were too variable to be reliably evaluated here.

When LAs were analyzed, laboratories using MD and CM-N_2 methods performed better than those with NA standards, because the isotope fractionation effects can be ignored. Most of the laboratories underestimated the true isotope values with LA, though the relative error was never more than 6%, which is in an acceptable range. To match the true at%^{15}N values of labeled materials, laboratories would need to use certified, ^{15}N-enriched international reference materials in a suitable range, ideally within the range of the samples, and utilize two- or more-point calibrations, an approach rarely adopted, even in well-known stable isotope laboratories for labeled samples.

Nearly all laboratories failed to provide comparable data for the isotopic composition of NO_3^- and NH_4^+ in the environmental samples with the variable methods utilized. Matrix effects and effects of "contaminating" compounds can be a problem for all methods, but also analytical errors and improper standardization procedures need to be considered. We conclude that the current reproducibility is not sufficient for meta-analysis of $\delta^{15}N$ of N_i at NA and provide guidelines to aid choosing the right method for particular samples and steps for optimization of standard operation procedures (SOP) (Figure 3). This interlaboratory comparison is a first step for future endeavors to develop harmonized protocols and best-practice guidelines for $\delta^{15}N$ measurements of N_i.

Though all the methods studied here have never been comparatively evaluated in one systematic interlaboratory test, the main differences between them are in agreement with prior tests of the individual methods[32–35] and reflect the historical development of the individual methods. The CM-N_2 methods (e.g., hypobromite oxidation) were the first in the field, and other methods were developed subsequently, to overcome their weaknesses, which are mostly related to their low precision and accuracy and the high sample need. The MD methods are known to have two overarching challenges, as discussed here and in other methods papers:[15,20,33,52,54] (a) the completeness of NH_4^+ collection from the sample into the acid traps, coupled with the strong isotope effect of this step, and (b) cross-reactivity by dissolved organic nitrogen in natural samples. These challenges were part of the motivation for the development of N_2O-based methods for NA isotopic analyses, the DN and the CM-N_2O methods, alongside with great improvements in the sensitivity for N_i analysis. Accordingly, the better performance of the N_2O-based methods is not surprising and indeed has been demonstrated previously, explaining why so much of the field has adopted these methods. This interlaboratory comparison was nevertheless novel and comprehensive, because (a) never before all the three method groups were compared simultaneously on the same set of samples, (b) both NO_3^- and NH_4^+ isotope analyses were performed in one study, (c) both NA and ^{15}N-enriched materials were investigated, (d) both reference materials and environmental samples were included in the interlaboratory comparison, and (e) by conducting the test as an the interlaboratory comparison, we examined the variability of the whole method chain and thus also the performance of laboratories. This allowed us to test thus whether $\delta^{15}N$ results from N_i produced by different laboratories using the same or a different method are intercomparable. Particularly, the latter two points revealed major, novel challenges and important new insights into the capability of commonly used IRMS methods to reproduce $\delta^{15}N$ of N_i in natural samples.

4 | CONCLUSIONS

Most laboratories using common methods to analyze ^{15}N in N_i produced acceptable results of $\delta^{15}N$ of N_i for standards in single forms. The accuracy of the results depends not only on the method used but also on the performance of the laboratory and the application and suitability of the method for the particular sample. The highly variable results of the environmental samples tested in this interlaboratory comparison suggest, however, large uncertainties in any $\delta^{15}N$ data reported from environmental samples analyzed by any of the widely used methods. It is difficult to relate the overall unsatisfying performance to singular factors, but given that many participating laboratories routinely analyze $\delta^{15}N$ of N_i compounds in environmental samples, these differences are of concern. Further systematic tests are immediately needed to test, evaluate, and optimize the methods compared in this interlaboratory comparison. In addition, the performance of laboratories conducting routinely $\delta^{15}N$ analysis of N_i must be improved. Before this careful examination, caution should be exercised when interpreting isotope data of N_i forms. The development of environmentally relevant standards, where sample matrices (e.g., river water, soil extracts) are first stripped from native NH_4^+ and NO_3^- by ion exchange or DN approaches, followed by addition of N_i of known isotopic composition, would greatly help to assess and compare the methods. In addition, it is highly recommended that laboratories include known

reference materials in their analysis and validate their measurements before sending out results. Regular participation in relevant proficiency tests and interlaboratory comparisons is strongly recommended. This should enable laboratories to detect poor performance as a result of mistakes during analytical runs.

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DATA AVAILABILITY STATEMENT

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SUPPORTING INFORMATION

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