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Validation of a dynamic ammonium extraction technique for the determination of ^{15}N at enriched abundances

Cristina Diaconu *, Natacha Brion, Marc Elskens, Willy Baeyens

Vrije Universiteit Brussel, Laboratory of Analytical and Environmental Chemistry, Pleinlaan 2, 1050 Brussels, Belgium

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Abstract

A diffusion method for extracting ammonium from marine, estuarine and fresh waters for $^{15}N/^{14}N$ isotopic ratio determinations at enriched level was developed and validated. The method is based on the conversion of NH_4^+ to NH_3 gas under alkaline conditions, diffusion of NH_3 out of the solution to the headspace, NH_3 trapping on an acidified GF/D glass fiber filter, and subsequent $^{15}N/^{14}N$ isotope ratio determination with mass spectrometry. The diffusion period necessary to extract sufficient N in order to accurately measure the atom% ^{15}N was reduced to less than 15 h by bubbling the sample with a carrier gas (Air) at room temperature. The technique uses 250 mL sample volume and enables accurate atom% ^{15}N measurements in NH_4^+ pools as small as 1.25 μM. A standard operating procedure for ammonium extraction is given involving method performance criteria, such as accuracy, precision, detection limit, quantification limit and robustness. The efficiency of the NH_4^+ extraction ranged from 40 to 100%. The quantification limit of the method was estimated at around 0.26 μmol% N, for an initial ^{15}N abundance of ∼1%. The within-laboratory reproducibility amounted to 0.03 atom% ^{15}N. It was shown that the recovery rate obtained after extraction of the certified reference material (CRM: IAEA-311) solution falls within the 95% confidence interval of the certified values. By applying the developed method to fortified natural water samples of different conductivities, the atom% ^{15}N determinations were precise and accurate for $\alpha = 1$–5%.

Keywords: ^{15}N; Ammonium; Diffusion; Method; Mass spectrometry

1. Introduction

Nitrogen exists in many forms and oxidation states in aquatic ecosystems, but ammonium (NH_4^+) has been regarded as a key intermediate in the marine nitrogen cycle [1]. As a highly labile nitrogen compound, ammonium rarely accumulates in the aquatic ecosystems, but comprises a relatively small pool with rapid turnover rates. Processes like uptake, remineralization, nitrification and transport determine the concentration and distribution of ammonium in a given location. Ammonium represents a major nitrogen source in sustaining phytoplankton growth [2–7], as well as the sole energy-yielding substrate for ammonium-oxidizing bacteria [8]. Ammonium is the first and most reduced nitrogenous end product resulting from complete degradation of organic material (ammonification process). Generally, ammonium is made available to phytoplankton through direct excretion by zooplankton or degradation of organic matter by microheterotrophs [1,6].

Providing quantitative information on the dynamics of the ammonium regeneration flux is essential for understanding phytoplankton nutrition and nitrogen cycling in aquatic ecosystems.

Our present knowledge of ammonium regeneration fluxes is largely based on studies using ^{15}N tracer techniques [9]. An essential step in this technique is the complete isolation of ammonium from the aqueous phase with a minimum expenditure of time, labour and hazardous chemicals and a highly diminished risk of contamination [10].

Several methods have been published for the extraction of dissolved ammonium for ^{15}N tracer studies in water [11,12] and soil [13]. However, these methods should be sensitive enough to allow accurate analysis of the often very small pools of NH_4^+ found in small samples [14]. Previous methods for isolating ammonium from aqueous samples prior to ^{15}N isotope ratio analysis included:

1. The extraction of ammonium by precipitation as a mercury compound [15] and by complexation in combination with

* Corresponding author. Tel.: +32 2 629 39 68; fax: +32 2 629 32 74.
E-mail address: cdiaconu@vub.ac.be (C. Diaconu).

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Nomenclature

$N_{initial}$ (μM) the initial ammonium concentration in the extraction solution
N (μmol) N quantity trapped on filter
E (%) efficiency of ammonium extraction
t (h) diffusion time
N_{sample} (μmol) the gross N content on the filter
N_{blk} (μmol) the mean N content of all the analytical blanks
atom $^{15}N\ \%_{net}$ the net atom% ^{15}N value of the N on the filter (after blank correction)
atom $^{15}N\ \%_{sample}$ the gross atom% ^{15}N value of the N on the filter corrected for trueness
atom $^{15}N\ \%_{blk}$ the mean atom% ^{15}N value obtained for all the analytical blanks
DL (μmol%) detection limit
QL (μmol%) quantification limit
CRM = IAEA-311 2.05% $(^{15}NH_4)_2SO_4$ with C.I. = 2.03–2.06%
95% C.I. 95% confidence limits of the certified value
$\sigma^2_{N_{net}\cdot{}^{15}N\,\%_{net}}$ the variance on $N_{net}\cdot{}^{15}N\ \%_{net}$
n number of extraction experiments
s_{WR} the within-laboratory reproducibility

solvent extraction to form the indophenol blue complex [16] or with solid phase extraction [17].
2. The cation exchange resin-based method for the extraction of ammonium from freshwater samples for on-line N-isotope ratio determination [18].
3. The distillation techniques: steam distillation [19,13], direct distillation [20,21] and distillation in combination with evaporation [11] or microdiffusion [22].
4. The diffusion procedures from Kjeldahl digests [23,24], soil extracts [25,26] and water samples [27–29].

The most popular techniques in recent years are the diffusion techniques. The advantages of the diffusion techniques over the other procedures include: (1) simplified sample preparation, as they require less operator skill and time [30]; (2) no cross-contamination between samples during the diffusion procedure, as disposable containers can be used.

The fundamental principles of the diffusion techniques, as outlined by Conway [31] are the following:

(1) Diffusion rates increase with increased pH, increased temperature, reduced depth of the sample solution, increased surface area of the sample solution and increased ionic strength of specific ions (e.g. K^+, Na^+, CO_3^{2-}, OH^-) in the sample solution.
(2) The rate-limiting processes in a diffusion system are movement of NH_3 through the liquid sample and movement across the gaseous layer above the sample.
(3) Diffusion analysis of ^{15}N samples is complicated by the sensitivity of the isotope technique to contamination or any process that causes isotopic fractionation [30].

The diffusion techniques have advantages and drawbacks that must be weighed in experimental applications with different sample species and sizes. However, a universal precaution that needs underscoring is the strict control of isotope fractionation, which could lead to the overestimation of the ^{15}N isotope dilution and corresponding biological mineralization. Therefore, the present diffusion methods require the complete separation of ammonium from the aqueous phase in which it occurs (soil extracts, water sample), since isotope fractionation has been reported for N recovery less than 100%. This is often achieved by treating the sample with MgO or KOH to convert ammonium ion (NH_4^+) to NH_3 (with or without addition of Devarda's alloy to reduce NO_3^- and NO_2^- to NH_4^+, and sulfamic acid to destroy NO_2^-), followed by the NH_3 diffusion into an acidified trap. The acid traps used to collect NH_3 during diffusion experiments consisted of vials of concentrated H_2SO_4, HCl or H_3BO_3 [32,23], capsules of acid-washed zeolite [26] or filter disks acidified with $KHSO_4$, H_2SO_4 or H_3BO_3 and suspended on stainless steel wire above the sample [25,33–37] or sealed inside Teflon tape for placement within the sample [38,37,27,28]. Diffusion procedures have been carried out in a variety of vessels, including glass digestion tubes [32], plastic specimen containers [25] and screw-cap bottles or canning jars [34]. An especially useful option is to carry out diffusions with gentle heating on a hot plate, which dramatically reduces the diffusion period [39], but this option cannot be used with a plastic specimen container.

Next, the isolated and trapped ammonium is oxidized into N_2 gas, which is finally isotopically analyzed using IRMS. The oxidation is performed either through high-temperature combustion in an elemental analyzer (EA) connected in-line with the IRMS instrument or by Rittenberg oxidation of the ammonium salt after evaporation as an off-line preparation step.

In this paper, we developed and validated a simple and efficient diffusion method for the extraction of ^{15}N-NH_4^+ at enriched level from water samples currently characterized by NH_4^+ concentrations down to 1.25 μM, within a short period of time (5–24 h), and its subsequent atom% ^{15}N determination, while avoiding measurable isotopic fractionation. The method involves a minimum amount of labour, hazardous chemicals and a greatly diminished risk of contamination. This analytical technique is applicable to a wide range of aquatic ecosystems, and therefore represents an important advance in evaluating nitrogen cycle in natural marine and freshwater bodies.

2. Materials and methods

2.1. Description of the ammonium extraction experiment

The ammonium extraction technique is based on the conversion of NH_4^+ to NH_3 gas under alkaline conditions (pH 13), diffusion of NH_3 out of the solution to the headspace, followed by the NH_3 trapping on an acidified (2.5 M $KHSO_4$) GF/D glass fiber filter, and its subsequent $^{15}N/^{14}N$ isotope ratio mass-spectrometric analysis. To speed up the procedure and maximize the extraction efficiency, the sample is degassed with air carrier gas through a flow regulating system.

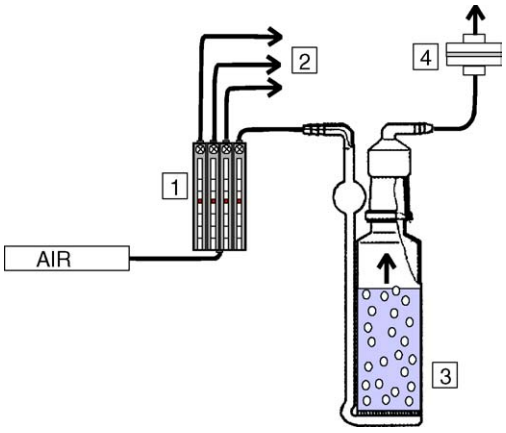

Fig. 1. Schematic representation of the ammonium extraction device: (1) flow regulator-meter system with floater connected to a pure air gas bottle; (2) silicone tubes connecting the flow regulating system to the gas-inlet of the diffusion bottle; (3) NH_4^+ diffusion bottle; (4) stainless steel in-line filter holder with an acidified GF/D filter (25 mm, glass fibre) connected to the gas-outlet of the diffusion bottles.

The NH_4^+ extraction device is presented in Fig. 1 and consists of one air (respirable air) gas bottle connected to a flow regulator system (Aalborg 150 mm tube, 3079 mL/min air; floater in sapphire + stainless steel flow regulator valve + stainless steel manifold four tubes holder system) allowing the parallel distribution of gas at four outlets with the same flow. Each outlet is connected with silicone tubes to the inlet of a glass diffusion vessel. The diffusion vessel consists of 350 mL gas-wash bottle with a glass filter plate bottom allowing air bubbling (Schott Duran gas-wash glass bottles, 350 mL). The gas outlet of each bottle is connected with silicone tubes to a 25 mm diameter stainless steel in-line filter holder (Gelman Laboratory, 25 mm diameter), holding each a 25 mm acidified GF/D glass fibre filter (Whatman). To avoid any ammonia gas loss out of the system during the diffusion procedure, PTFE (Teflon) tape is wrapped around all connections.

The general extraction procedure is as follows:

All material used for the extraction is first decontaminated with 5% H_2SO_4 solution and rinsed with ultrapure deionized water. The GF/D glass fibre filters are decontaminated by heating at 450 °C during 8 h.

A known volume (250 mL) of sample is put in every extraction vessel and alkalinized with the addition of 1.25 mL of 80% KOH (pH is raised up to 13). Immediately, the diffusion bottles are closed hermetically. Compressed pure air is then injected from the (air) gas bottle into the flow controller and distributed with equal flows (1210 mL/min) to the gas inlets of the four extraction vessels. The air passes through the porous glass-fiber disc, bubbles in the solution and entrains the dissolved NH_3 out to the headspace and further towards the acidified filter. There, the NH_3 gas reacts with the acidic solution (50 μL of 2.5 M $KHSO_4$ or 30 μL of 4 M H_2SO_4) to form $(NH_4)_2SO_4$ salt that is trapped on the filter. After the diffusion experiment, the filter is placed in a clean plastic Petri dish, dried in an oven at 50 °C for 8 h and analysed for its N-content and atom% ^{15}N abundance using an Elemental Analyzer-Isotope Ratio Mass Spectrometer (EA-IRMS) system (see details below). Ammonium concentration is also measured in the remaining solution according to the indophenol blue complexation method [40]. The standards are prepared at the same pH as the extracted solution.

2.2. Performed extraction experiments

1. Blank extraction experiments: Analytical blanks, using ultrapure deionized distilled water as diffusion solution and containing all reagents, were processed in the same way as the samples, i.e. from sample pre-treatment up to the measurement. In this way, any N contamination occurring during the extraction experiment can be taken into account.
2. Effect of diffusion time: The impact of the diffusion time on the extraction efficiency and on the ^{15}N abundance measurement was assessed. Extraction experiments were performed in triplicate for each time period (5, 10, 15 and 24 h) using 250 mL of 5 μM, 1.15% $^{15}NH_4^+$ enriched standard solutions.
3. Effect of NH_4^+ concentration: A series of $^{15}NH_4^+$ extraction experiments were performed in order to assess the effect of NH_4^+ concentration on the extraction efficiency and on the ^{15}N abundance determination. For this purpose, six replicates of 250 mL $^{15}NH_4^+$ standard solutions were prepared, and subsequently submitted to diffusion for 15 h at room temperature. The ammonium concentration was 0.63, 1.25, 2.5, 5.0, 8.5 and 10 μM and the ^{15}N abundance was 1.15%.
4. Diffusion on certified reference material: To test the accuracy of the ammonium extraction technique, six replicate diffusions were performed on 250 mL of 8.52 μM, 2.05% $^{15}NH_4^+$ enriched solutions prepared by dissolving 0.00112 g of 2.05% certified reference material (CRM = IAEA-311, 2.05% $(^{15}NH_4)_2SO_4$ with 95% C.I. = 2.03–2.06%) in 2000 mL ultrapure deionized water.
5. Effect of the trapping agent (2.5 M $KHSO_4$ and 4 M H_2SO_4): To test the influence of the trapping agent used in the acidification of the GF/D filters on the efficiency of NH_4^+ extraction and on the ^{15}N abundance determination, two series of experiments were performed, respectively, with 2.5 M $KHSO_4$ [41,37,42] and 4 M H_2SO_4 [22,35,27–29]. The experiments were performed on six replicates of 250 mL of 5 μM, 1.15% $^{15}NH_4^+$ enriched solutions for the test with 2.5 M $KHSO_4$ and on eight replicates of 250 mL of 5 μM, 1.15% $^{15}NH_4^+$ enriched solutions for the test with 4 M H_2SO_4. All $^{15}NH_4^+$-enriched solutions were allowed to diffuse for 15 h at room temperature.
6. Extraction of fortified estuarine water samples: The developed technique was tested on a series of estuarine water samples collected during a spring season (April 2003). The sampling stations were selected by following a conductivity gradient, with values ranging from 964 to 14,050 μS/cm, in the Scheldt estuary (Belgium–The Netherlands). Prior to extraction, the water samples were analysed for their original ammonium concentration [40]. Dilutions of samples were then performed to adjust the final ammonium concentration around 10 μM, prior to ^{15}N enrichment (see Table 1). At each station, 1 L of water sample (previously diluted, if necessary) were taken and spiked with different volumes (0.5–1 mL) of 100 μM, 99% ^{15}N-NH_4^+ solution. Next, duplicates of

Table 1
Dilutions performed on the estuarine water samples in order to reach ammonium concentrations of around 10 μM prior to their submission to the extraction procedure

Conductivity (μS/cm)	$[NH_4^+]_{ambient}$ (μM)	Dilution factor	$[NH_4^+]_{diluted}$ (μM)	Amount (mL) of spike[a] added
964	109	10	10.9	0.83
1166	106	8	13.3	0.84
1540	102	10	10.2	0.8
3610	60.2	5	12	1
10450	14.8	No dilution	14.8	1
14050	5.9	No dilution	5.9	0.5

[a] 100 μM, 99% $^{15}NH_4^+$.

250 mL of each sample were transferred to the extraction vessels and extracted for NH_4^+ during 5 h at room temperature.

For each performed experiment, we define the efficiency of ammonium extraction E (%) as:

$$E\,(\%) = 100 \cdot \frac{N_{trapped}}{N_{initial}} \quad (1)$$

2.3. Determination of N (μmol) content and atom% ^{15}N of the NH_4^+ recovered on the GF/D filter

A Thermo Finnigan delta + XL (continuous flow type) mass-spectrometer with standard set up for N_2 gas, interfaced with a Flash Carlo Erba NA1500 C/N analyser via a Conflo III interface was used for performing the atom% ^{15}N and N quantity analysis of the ammonia recovered on the dried GF/D filters. Several measures have been taken to prevent corrosion of the reduction column, as well as background contamination due to the acid trapping agent used on the GF/D filters:

(1) Ag capsules were used to fold the dried GF/D filters prior to the mass spectrometric analysis.
(2) Silver wool has been added to the reduction column to neutralize the sulphur oxides formed.
(3) Water and carbon traps were attached between the reduction column and the GC column to prevent moisture and carbon-compounds contamination during the mass spectrometric analysis.

The temperatures of the reduction and combustion columns were set at 1040 and 640 °C, respectively.

For each preparation cycle, a series of blanks including the empty Ag capsules, acidified filter blanks and analytical blanks (obtained by extracting deionized distilled water) were prepared and used for blank corrections.

Gross N content of filter samples were determined from N peak area by comparison to a calibration curve obtained by weighing appropriate quantities (0, 0.1, 0.2, 0.4 and 0.6 mg) of CRM IAEA-311 2.05% $(^{15}NH_4)_2SO_4$ using a Sartorius microbalance. The peak areas measured for the standards were each corrected for the mean cup blank peak area in order to avoid influence of cup blanks on the N determination. To determine the quantity of N (μmol) recovered on GF/D filter after the extraction, the gross N content is corrected for the mean N content of all analytical blanks.

Measured gross ^{15}N % of filter samples were first corrected for the deviation between atom% ^{15}N abundance given by the machine and true atom% ^{15}N value. This was done by comparison to a calibration curve relating "measured machine" atom% ^{15}N with true atom% ^{15}N, using CRM's of different abundances (IAEA-311 corresponding to 2.05% $(^{15}NH_4)_2SO_4$ and IAEA-N1 corresponding to 0.36% $(NH_4)_2SO_4$). Finally, to determine the ^{15}N % of N recovered on GF/D filters after the extraction, the gross ^{15}N signal is corrected for the mean N content and ^{15}N % of all analytical blanks according to the isotope dilution law (Eq. (2)):

$$^{15}N\,\%_{net} = \frac{N_{sample} \cdot {}^{15}N\,\%_{sample} - N_{blk} \cdot {}^{15}N\,\%_{blk}}{N_{sample} - N_{blk}} \quad (2)$$

where N_{sample} is the the gross N (μmol) content on the filter; N_{blk} the mean N (μmol) content of all the analytical blanks = 0.14 μmol (see below); $^{15}N\,\%_{net}$ the net atom% ^{15}N value of the N on the filter (after blank correction); $^{15}N\,\%_{sample}$ the gross atom% ^{15}N value of the N on the filter corrected for trueness; $^{15}N\,\%_{blk}$ is the mean atom% ^{15}N value obtained for all the analytical blanks = 0.364% (see below).

3. Results

3.1. Blank extraction experiments

To account for the N contamination occurring during the diffusion procedure, the dried analytical blank GF/D filters, obtained by extracting ultrapure deionized water (DDW) were analyzed for the N amount and ^{15}N abundance. The results, plotted in Fig. 2A and B, indicate that possible N contamination could lead to extreme values (outliers), which are not representative for the rest of data.

3.2. Effect of diffusion time

The results of the tests investigating the relationships between diffusion time, extraction efficiency and atom% ^{15}N of extracted NH_4^+ are plotted in Fig. 3A and B. Extraction efficiency increases from 40% (S.D. = 2) at 5 h to 112% (S.D. = 4) at 24 h, reaching a plateau when diffusion was carried out for more than 15 h (Fig. 3A). The ^{15}N abundance obtained after 5 h of diffusion averaged 1.27% (S.D. = 0.004), significantly higher than the nominal value of 1.15%. As the diffusion time increased from 10 to 24 h, the mean values reached a plateau, ranging from 1.21%

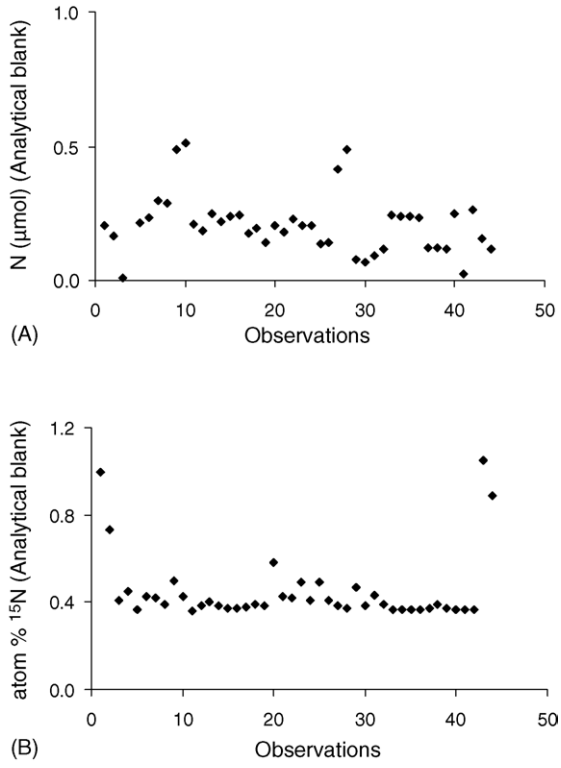

Fig. 2. Variation of (A) the quantity (μmol-N) corresponding to the amount of N trapped on blank GF/D filters and (B) the abundance (atom% ^{15}N) for all (N = 44) ammonium extraction experiments carried out at both enriched and natural abundances.

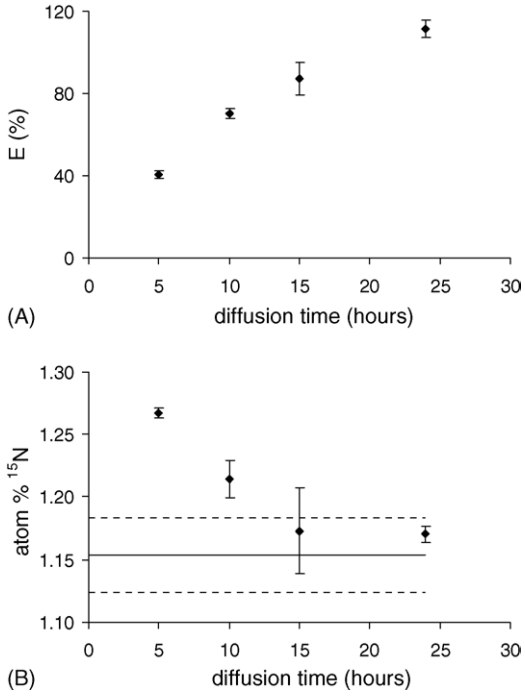

Fig. 3. Variation with the diffusion time of (A) the mean extraction efficiency E (%) and (B) the mean atom% ^{15}N abundance for an extraction experiment carried out with a 5 μM, 1.15% ^{15}N-NH_4^+ standard solution. The error bars corresponding to the 5, 10, 15 and 24 h extractions were calculated on three replicates. The continuous line represents the atom% ^{15}N nominal value and the broken lines (bottom) the uncertainity on the atom% ^{15}N nominal value under repeatability conditions (1.15% ± s_{WR}). s_{WR} is the within-laboratory reproducibility on atom% ^{15}N determinations = 0.03 atom% ^{15}N.

(S.D. = 0.01) to 1.17% (S.D. = 0.01) closer to the nominal value. To reach a plateau in the determination of both the N amount trapped on the GF/D filter and the atom% ^{15}N, a time period of at least 15 h seems to be required when diffusing a $^{15}NH_4^+$ enriched solution of 5 μM and 1.15%.

3.3. Effect of NH_4^+ concentrations

A series of experiments were carried out to investigate the effects of the ammonium concentration on the efficiency of extraction and on the recovery of ^{15}N abundance. The extraction was performed with a range of 0.63–10 μM ammonium concentrations, while holding the diffusion time at 15 h and ^{15}N abundance at 1.15%. The results of this study, plotted in Fig. 4A, showed that the efficiency of extraction increased with increasing NH_4^+ concentration. Overall, extraction efficiencies were higher than 87%, varying between 87% (S.D. = 8) and 110% (S.D. = 5) for NH_4^+ concentrations in the range of 2.5–10 μM. In contrast herewith, extraction efficiency was only 25% (S.D. = 4) for NH_4^+ concentration lower than 1.3 μM.

When analysing the atom% ^{15}N content (Fig. 4B), the mean values obtained for six replicate enriched solutions with ammonium concentrations ranging from 2.5 to 10 μM are very close to the nominal value of 1.15%. On the contrary, for ammonium concentration lower than 1.3 μM, the measured atom% ^{15}N was much higher than the nominal value.

3.4. Trapping agent

Two trapping agents, 2.5 M $KHSO_4$ and 4 M H_2SO_4 were compared to investigate their efficiency in trapping ammonia on GF/D filter. For this purpose, replicates of 5 μM, 1.15% $^{15}NH_4^+$ enriched solutions were extracted for 15 h at room temperature. The results, plotted in Fig. 5A and B, show that slightly higher $^{15}NH_4^+$ recoveries on the filter were obtained when using 2.5 M $KHSO_4$ rather than 4 M H_2SO_4. The 2.5 M $KHSO_4$ trapping agent has been therefore preferred to 4 M H_2SO_4 as it is also less corrosive to the Ag capsules and the IRMS system.

4. Discussion: method validation

4.1. Analytical blanks

Each batch of diffusion experiments (four bottles in parallel) included a blank diffusion experiment (using ultrapure deionized water as a diffusion solution). The results of these experiments (Fig. 2A and B) were treated in order to determine the mean and standard deviation of the N amount and atom% ^{15}N associated with the analytical blank. In this way, the result of all possible N sources, contaminating samples along the ammonium extraction procedure, could be assessed with control charts. Outliers or at least extreme values that deserve close scrutiny were first identified by using interquartile range approach [43]. This procedure does not require any assumption, and therefore is robust

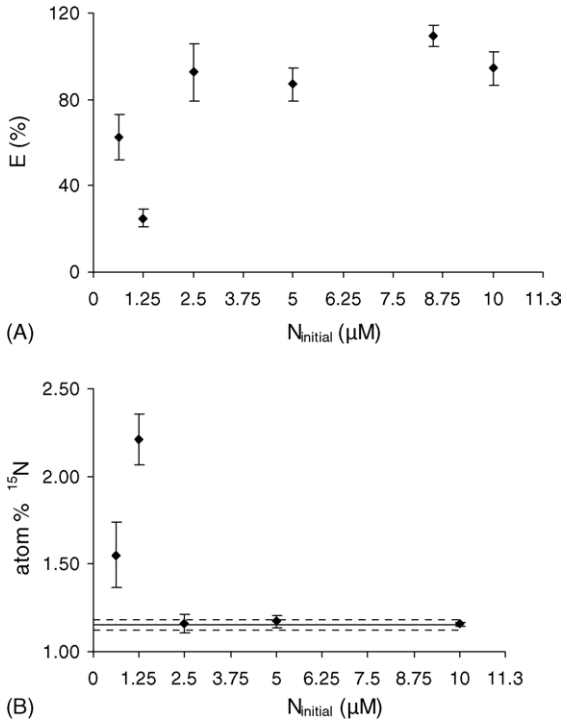

Fig. 4. Variations of (A) mean extraction efficiency E (%) and (B) mean atom% ^{15}N abundance with a series of ammonium standard concentrations (0.63–10 μM, extraction time = 15 h, ^{15}N abundance = 1.15%). The error bars corresponding to each of the ammonium standard concentrations (0.63–10 μM) were calculated on six replicates. The continuous line represents the atom% ^{15}N nominal value and the broken lines (bottom) the uncertainity on the atom% ^{15}N nominal value under repeatability conditions (1.15% ± s_{WR}). s_{WR} is the within-laboratory reproducibility on atom% ^{15}N determinations = 0.03 atom% ^{15}N.

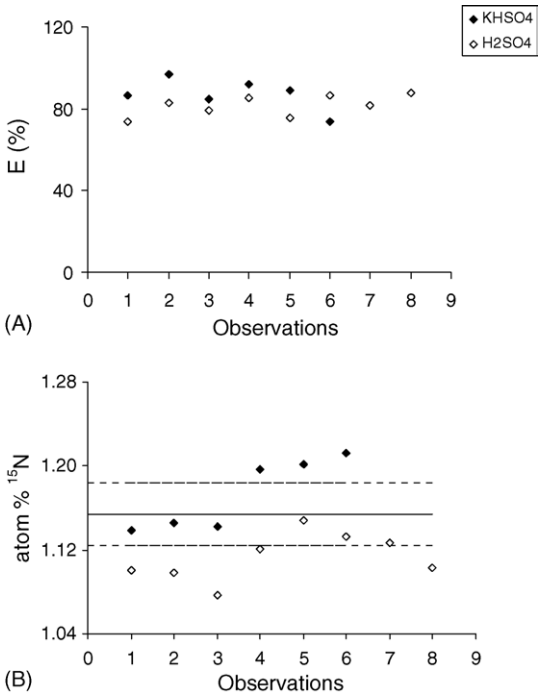

Fig. 5. Variations of (A) mean extraction efficiency E (%) and (B) atom% ^{15}N abundance for a series of extractions (5 μM, 1.15% ^{15}N-NH_4^+ standard solution). Comparison of two trapping agents: 2.5 M $KHSO_4$ and 4 M H_2SO_4. The continuous line represents the atom% ^{15}N nominal value and the broken lines (bottom) the uncertainity on the atom% ^{15}N nominal value under repeatability conditions (1.15% ± s_{WR}). s_{WR} is the within-laboratory reproducibility on atom% ^{15}N determinations = 0.03 atom% ^{15}N.

[44]. It indicated that blank values beyond −0.04/0.41 (μmol) and 0.3/0.5 (%) can be regarded as extreme and cancelled.

After outlier removals, it can be shown that the N amount trapped on the GF/D filter is normally distributed ($P > 0.2$) with mean N = 0.14 μmol and S.D. = 0.07 μmol ($n = 44$), while the atom% ^{15}N distribution remains skewed. With values ranging from 0.37 to 0.43% in the fourth spread (IQR) (Fig. 2B), it is clear that the abundance of the analytical blanks shows a contamination with enriched ^{15}N. This contamination is probably related to the fact that after the extraction procedure, blank filters were dried in an oven side-to-side to extraction filters enriched in ^{15}N-NH_4^+. Cross-contamination from a ^{15}N-enriched filter to a blank filter, is therefore possible. Better results were obtained by taking into account analytical blank experiments conducted only with non-enriched samples (Fig. 6). Under these conditions, the data appears to be normally distributed ($P > 0.2$) with mean atom% ^{15}N = 0.36% and S.D. = 0.005% ($n = 11$).

Summarizing, the average quantity of N and its ^{15}N isotopic signal obtained for the analytical blanks are 0.14 (S.D. = 0.07) μmol N and 0.36% (S.D. = 0.005). These values were used to correct our measurements according to Eq. (2).

An overview of nitrogen blank contamination associated with diffusion ammonium extraction methods previously reported in literature is given in Table 2. Compared to other diffusion extraction techniques, contamination arising during the sample handling is in the lower range, close to the one reported for the technique developed by Sigman et al. [27].

4.2. Detection and quantification limits

Detection and quantification limits of the described method refer to the smallest N (μmol) quantity needed to be trapped on GF/D filter in order to accurately determine the ^{15}N abundance. Because the blank correction is performed by subtracting the

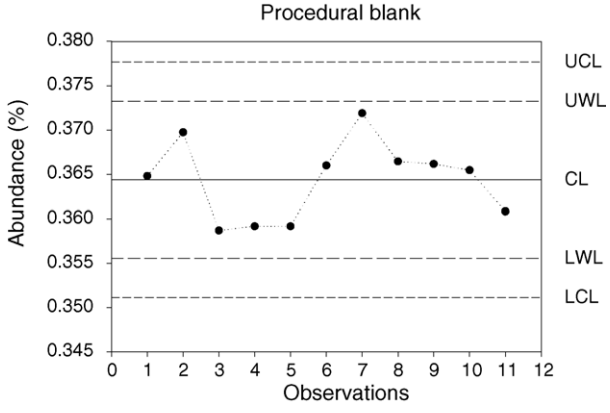

Fig. 6. Control chart showing variation of the analytical blank atom% ^{15}N values of N trapped on the GF/D filters for a number of blank NH_4^+ diffusion experiments, carried out in parallel with diffusion experiments at natural (non-enriched) abundance.

Table 2
Overview of the nitrogen blank contamination associated with ammonium extraction methods previously reported in literature

Reference	Sample type	Reported N blank for 250 mL sample (μmol)
This work (2005)	DDW	0.14
Kristiansen and Paasche [7]	Coastal, oceanic water	0.12
Sorensen and Jensen [38]	2 M KCl solution	0.28
Lory and Russelle [35]	Kjeldahl digest	2.3
Sigman et al. [27]	Seawater	0.1
Holmes et al. [28]	DDW	0.01

mean of n blank determinations, the variance on $N_{net}.{}^{15}N\,\%_{net}$ is:

$$\sigma^2_{N_{net}.{}^{15}N\,\%_{net}} = \frac{\sigma^2_{N_{sample}.{}^{15}N\,\%_{sample}} + \sigma^2_{N_{blk}.{}^{15}N\,\%_{blk}}}{n} \quad (3)$$

where $\sigma^2$is the variance and n is the number of blank experiments.

If the standard deviation is independent of the quantity (homoscedasticity):

$$\sigma_{N_{net}.{}^{15}N\,\%_{net}} = \sigma_{N_{blk}.{}^{15}N\,\%_{blk}} \cdot \sqrt{\frac{n+1}{n}}$$

$$= N_{blk} \cdot {}^{15}N\,\%_{blk} \cdot \sqrt{\frac{n+1}{n} \cdot \left(\left(\frac{\sigma_{N_{blk}}}{N_{blk}}\right)^2 + \left(\frac{\sigma_{{}^{15}N\,\%_{blk}}}{{}^{15}N\,\%_{blk}}\right)^2\right)} \quad (4)$$

When the sample does not contain the analyte, $N_{sample}.{}^{15}N\,\%_{sample} = N_{blk}.{}^{15}N\,\%_{blk}$ and their difference follows a normal distribution with a population mean of zero and a standard deviation:

$$\sigma_0 = \sigma_{N_{blk}.{}^{15}N\,\%_{blk}} \sqrt{\frac{n+1}{n}} \quad (5)$$

For blank corrected measurements, detection (DL) and quantification (QL) limits (μmol%) are then given by IUPAC [45]:

$$DL = 2 \cdot t_{n-1,95\%} \cdot \sigma_0 + 3.3 \cdot \sigma_0 \; (n > 30) \quad (6)$$

$$QL = 6 \cdot t_{n-1,95\%} \cdot \sigma_0 + 10 \cdot \sigma_0 \; (n > 30) \quad (7)$$

In our case, $\sigma_0 = 0.37$, and thus DL = 0.09 μmol% and QL = 0.26 μmol%. These results imply that the minimum amounts of nitrogen trapped on GF/D filter for quantitative atom% ^{15}N determinations should be around 0.71 μmol when working at the natural background level (∼0.365%), 0.50 μmol when working at ∼0.5% enriched background level and 0.29 μmol when working at ∼1% enriched background level. This amount decreases drastically to less than 0.07 μmol in tracer enrichment and dilution experiments, where initial ^{15}N abundance should range between 5 and 10%. Knowing that the average efficiency of extraction is around 90% for 15 h extraction of 250 mL solutions (Fig. 3A), we can reasonably estimate that samples with NH_4^+ concentrations down to 3.1 μM (0.36%), 2.2 μM (0.5%) and 1.3 μM (1%) will be suitable for extraction with the developed technique. Compared to other techniques (see Table 3) the developed method proved to be quite sensitive as concentrations down to 1.3 μM can be extracted successfully.

Table 3
Minimum N (μM) quantities necessary to be present in the original extraction solution, as reported by different ammonium extraction methods

Reference	Range of ^{15}N % abundances	Minimum N quantity required (μM)
This work (2005)	1%	1.3 μM in solution
This work (2005)	0.5%	2.2 μM in solution
This work (2005)	0.36%	3.1 μM in solution
Brooks et al. [25]	–	28.6 μM in solution
Liu and Mulvaney [33]	0.5%	14.3 μM N in soil extract
Holmes et al. [28]	Up to 100‰	5 μM in solution
Sigman et al. [27]	0.36%	5 μM in solution
Sebilo et al. [29]	0.3–39.6‰	14.3 μM in solution

4.3. Precision study

We have estimated the within-laboratory reproducibility and repeatability in a single experiment set-up using ANOVA analysis [43]. For this purpose, we used the data from the extraction of enriched $^{15}NH_4^+$ solutions with concentration in the range of 2.5–10 μM.

ANOVA table (see Appendix A) indicates that the differences in the mean values among the concentration groups are not statistically significant ($P = 0.332$). ANOVA table learns also that the residual mean squares, which can be termed here the mean squares within days is 0.00107. This represents a standard deviation s_r of 0.03% under repeatability conditions. Moreover, according to Eq. (8), the within-laboratory reproducibility s_{WR} was estimated at around 0.03 atom% ^{15}N:

$$\begin{cases} s_{WR} = \sqrt{s_r^2 + s_{between}^2} \\ s_{between}^2 = \dfrac{MS_{between} - MS_{within}}{N} \end{cases} \quad (8)$$

where s_{WR} is the within-laboratory reproducibility = 0.03 atom% ^{15}N; s_r the standard deviation under repeatability conditions = 0.030%; $s_{between}$ the standard deviation between concentration groups, $MS_{between}$ the mean squares between concentration groups days, MS_{within} the mean squares within days = 0.00107 and N is the number of extraction experiments carried out within a concentration group.

4.4. Accuracy study

Accuracy of the method was first tested by performing extraction with enriched solutions prepared from certified reference material IAEA-311 (2.05% $(^{15}NH_4)_2SO_4$). According to the BCR [1986] recommendations [46], we verified that (see Appendix A) (1) the standard error of the mean we obtained (S.E. = 0.003%) is less than the standard deviation of the distribution of certifying laboratory mean values (S.D. = 0.008%) and (2) the mean atom% ^{15}N value we obtained (2.06%) falls

Table 4
Extraction experiments carried out on IAEA-311 (CRM) NH_4^+ solutions, spiked standard NH_4^+ solutions and spiked natural samples to test the accuracy of the method

Type sample	N_{intial} (μM)	$N_{trapped}$ (μmol) (mean ± S.D.)	Remark	E (%) (mean ± S.D.)	$^{15}N_{initial}$ %	$^{15}N_{final}$ % (mean ± s_{WR})	Status
CRM (IAEA-311)	8.5	2.3 ± 0.1	>QL	110 ± 5	2.05	2.06 ± 0.03	Unbiased
Spiked standard solutions	0.63	0.10 ± 0.02	<QL	62 ± 11	1.15	1.55 ± 0.03	Biased
	1.3	0.10 ± 0.01	<QL	25 ± 4	1.15	2.21 ± 0.03	Biased
	2.5	0.6 ± 0.1	>QL	93 ± 13	1.15	1.16 ± 0.03	Unbiased
	5	1.1 ± 0.1	>QL	87 ± 8	1.15	1.17 ± 0.03	Unbiased
	10	2.4 ± 0.2	>QL	94 ± 8	1.15	1.16 ± 0.03	Unbiased
Spiked natural samples							
Conductivity (μS/cm)							
964	11	1.6 ± 0.1	>QL	53 ± 5	1.11	1.10 ± 0.03	Unbiased
1166	13	1 ± 0	>QL	27 ± 0	0.99	1.38 ± 0.03	Unbiased
1540	10	1.4 ± 0	>QL	49 ± 1	1.13	1.15 ± 0.03	Unbiased
10450	15	2.2 ± 0.3	>QL	54 ± 8	1.03	1.04 ± 0.03	Unbiased
14050	6	1.1 ± 0	>QL	62 ± 3	1.20	1.16 ± 0.03	Unbiased

^{15}N abundance after extraction is compared to initial ^{15}N abundance of the solution with t-tests. $N_{initial}$ (μM) is the initial NH_4^+ concentration, $N_{trapped}$ (μmol) the N trapped on the filter after extraction, E (%) the efficiency of extraction, $^{15}N_{initial}$% the initial atom% ^{15}N in the solution and $^{15}N_{final}$ % is the final atom% ^{15}N obtained after extraction. The quantification limit (QL) is 0.14 μmol.

within the 95% confidence limits of the certified value, which is 2.03–2.06%. Under the experimental conditions of the extraction (15 h diffusion) and for a restricted concentration range (8.5 μM of $(^{15}NH_4)_2SO_4$ at 2.05%), our method fulfils, therefore, the BCR requirements (see Table 4). Being limited by the CRM availability, we then treated the results obtained with spiked standard solutions over a wide range of NH_4^+ concentrations (0.63–10 μM). Herein, the accuracy was tested by comparing the mean obtained for the final atom% ^{15}N with the nominal value of the standard solution (1.15%) by means of t-tests and under repeatability conditions. Results are shown in Table 4. Overall, it can be concluded that there are no significant differences between the measured and nominal values except when the ammonium concentration is lower than 1.3 μM, i.e. when the N trapped on the filter is lower than the quantification limit (see previous section).

Similar test was finally carried out on spiked natural samples also called fortified samples. Table 4 indicated that for a level of significance of 1%, the ^{15}N isotope ratio determinations were all satisfactory. There is not a statistically significant difference between the nominal and measured atom% ^{15}N values, proving that the developed method can be successfully applied to natural water samples.

4.5. Ruggedness study

The term ruggedness or robustness was introduced by Youden and Steiner [47] into analytical chemistry. The Association of Official Analytical Chemists (AOAC) recommends that for each factor one defines a nominal and an extreme level. The nominal level is the level given in the procedure or the most probable level of an implicit factor, the extreme level is the one, which exceptionally might be attained in practice. For instance, from Fig. 3, the nominal level for the diffusion time being 15 h, one could consider that the extreme are 10 and 24 h and try to determine the effect on a response between those two levels. From Appendix A it can be concluded that there are not significant differences ($P = 0.158$) between the nominal value of the standardized solution (1.15%) and the measurements for extraction time ranging from 10 to 24 h and initial concentration higher than 2.5 μM. This proves that the developed method is robust when applied under the described conditions.

5. Conclusions

To conclude, the ammonium extraction technique hereby developed uses a standardized, simple and relatively cheap experimental setup (diffusion bottles, flow regulators, filter holders, glass-fibre filters and compressed air bottles). More importantly, it ensures a full control of the extraction process by monitoring the final NH_4^+ concentration in solution and the amount of N trapped on the filter, at the end of the diffusion experiment. The ammonium extraction experiments are successfully conducted at room temperature, by using a small and constant sample volume (250 mL) and extraction times' not exceeding 15 h. This is an advantage to previously described methods that often used much longer diffusion times. For example, in the diffusion method of Brooks et al. [25], quantitative recovery of NH_4^+ from KCl solutions containing between 80 and 800 μg N was obtained by diffusing for 6 days, at room temperature. In the diffusion method developed by Sigman et al. [27], samples were incubated for 4 days or longer. Overall, the efficiency of the NH_4^+ extraction ranged from 40 to 100%. Analytical blanks, which have been taken through the whole procedure from the sample pre-treatment up to the measurement, yielded a mean N quantity trapped on the GF/D filter of 0.14 μmol (S.D. = 0.07 μmol; $n = 44$) and a mean atom% ^{15}N of 0.364% (S.D. = 0.004%; $n = 11$). The detection limits of the developed method are of 0.7 μmol for an abundance of 0.36%, 0.5 μmol for an abundance of 0.5% and 0.3 μmol for an abun-

dance of 1%. For an extraction experiment conducted over a period of 15 h, these correspond to NH_4^+ concentrations in solution of 3.1 μM (0.36%), 2.2 μM (0.5%) and 1.3 μM (1%). These values are rather low compared to previously described methods (see Table 3). The method has thus the advantage of being applicable to a wide range of aquatic ecosystems (marine and freshwater ecosystems), and therefore represents an important advance in assessing the natural aquatic nitrogen cycle. The tests carried out on fortified natural samples of different conductivities demonstrated the accuracy and precision of our method when determining the ^{15}N abundances for $\alpha = 1$–5%, a diffusion time of 15 h and solutions characterized by ammonium concentrations in the range of 1.3–10 μM. So, for samples with higher NH_4^+ concentration, a dilution should be applied before the ^{15}N spiking. On the contrary, samples with lower concentrations should be spiked with known amounts of ^{15}N-NH_4^+ in order to reach an NH_4^+ concentration that satisfies the method requirements.

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Appendix A

ANOVA analysis applied to the experimental results as part of the method validation

Accuracy of the method (CRM: IAEA-311)[a]

CRM data. Confidence limits (5%–95%)	*N* (number of replicates)	Maximum abundance (^{15}N %)	Minimum abundance (^{15}N %)	Mean abundance (^{15}N %)	Standard deviation (^{15}N %)
2.03–2.06	6	2.053	2.074	2.06	0.01

Precision of the method (1.15% standard)[a,b]

Concentration group, *C* (μM)	*N* (number of replicates)	Maximum abundance (^{15}N %)	Minimum abundance (^{15}N %)	Mean abundance (^{15}N %)	Standard deviation (^{15}N %)
2.5	6	–	–	1.159	0.05
5	6	–	–	1.173	0.03
10	6	–	–	1.157	0.01

Ruggedness of the method (1.15% standard)[a,c]

Diffusion time (h)	*N* (number of replicates)	Maximum abundance (^{15}N %)	Minimum abundance (^{15}N %)	Mean abundance (^{15}N %)	Standard deviation (^{15}N %)
10	3	–	–	1.214	0.02
15	6	–	–	1.173	0.03
24	3	–	–	1.170	0.01

[a] Our results.
[b] The within-laboratory reproducibility (s_{WR}), over the three concentration groups is equal to 0.0332. As a consequence, the three mean atom% ^{15}N are not statistically different ($P = 0.332$).
[c] The conclusion is that the mean values for each diffusion time are not statistically different from the nominal value (1.15%) of our standard extraction solutions ($P = 0.158$).

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