IAEA TECDOC SERIES

IAEA-TECDOC-1850

IAEA-TECDOC-1850

# Sampling and Isotope Analysis of Agricultural Pollutants in Water


Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

IAEA
International Atomic Energy Agency

# SAMPLING AND ISOTOPE ANALYSIS OF AGRICULTURAL POLLUTANTS IN WATER

The Agency's Statute was approved on 23 October 1956 by the Conference on the Statute of the IAEA held at United Nations Headquarters, New York; it entered into force on 29 July 1957. The Headquarters of the Agency are situated in Vienna. Its principal objective is "to accelerate and enlarge the contribution of atomic energy to peace, health and prosperity throughout the world".

IAEA-TECDOC-1850

# SAMPLING AND ISOTOPE ANALYSIS OF AGRICULTURAL POLLUTANTS IN WATER

PREPARED BY THE
JOINT FAO/IAEA DIVISION OF NUCLEAR TECHNIQUES IN FOOD AND AGRICULTURE

INTERNATIONAL ATOMIC ENERGY AGENCY
VIENNA, 2018

# COPYRIGHT NOTICE



For further information on this publication, please contact:

Soil and Water Management and Crop Nutrition Section
International Atomic Energy Agency
Vienna International Centre
PO Box 100
1400 Vienna, Austria
Email: Official.Mail@iaea.org


Printed by the IAEA in Austria
August 2018

**IAEA Library Cataloguing in Publication Data**

Names: International Atomic Energy Agency.
Title: Sampling and isotope analysis of agricultural pollutants in water / International Atomic Energy Agency.
Description: Vienna : International Atomic Energy Agency, 2018. | Series: IAEA TECDOC series, ISSN 1011–4289 ; no. 1850 | Includes bibliographical references.
Identifiers: IAEAL 18-01175 | ISBN 978–92–0–105018–2 (paperback : alk. paper)
Subjects: LCSH: Stable isotopes in pollution research. | Nutrient pollution of water. | Water — Analysis. | Water — Composition.

## FOREWORD

The IAEA and the Food and Agriculture Organization of the United Nations (FAO), through the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, provide support to Member States for sustainable agricultural development through the use of isotope and nuclear techniques. The Joint Division's programme and activities focus on advancing soil, water and crop management technologies and practices for sustainable agricultural intensification.

Nutrient pollution associated with agricultural activities is a worldwide problem. Advances in stable isotope techniques can play a major role in identifying the sources of nutrient pollution in water. This knowledge of the pathways of contaminants in nature is in turn essential in the management of agricultural resources.

Appropriate sampling and sample preparation are the first steps to ensure the quality of stable isotope analysis for tracing water pollution. The standard operating procedures presented in this publication provide comprehensive instructions for surface water sampling and management, and for using the bacterial denitrifier and laser spectroscopy method to determine $\delta^{15}N$ and $\delta^{18}O$ composition of nitrate in water and the microdiffusion method to measure enriched $\delta^{15}N$ signatures in ammonium and nitrate.

This publication is targeted to scientists, technicians and students responsible for implementing procedures to collect and prepare water samples for isotope analysis. This step-by-step guidance will enable Member States to better determine the sources and flow paths of water contamination for improved water and nutrient management practices.

The IAEA wishes to thank all the contributors involved in the preparation of this publication. The IAEA officers responsible for this publication were G. Dercon, L. Heng and M. Heiling of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

*EDITORIAL NOTE*

*This publication has been prepared from the original material as submitted by the contributors and has not been edited by the editorial staff of the IAEA. The views expressed remain the responsibility of the contributors and do not necessarily represent the views of the IAEA or its Member States.*

*Neither the IAEA nor its Member States assume any responsibility for consequences which may arise from the use of this publication. This publication does not address questions of responsibility, legal or otherwise, for acts or omissions on the part of any person.*

*The use of particular designations of countries or territories does not imply any judgement by the publisher, the IAEA, as to the legal status of such countries or territories, of their authorities and institutions or of the delimitation of their boundaries.*

*The mention of names of specific companies or products (whether or not indicated as registered) does not imply any intention to infringe proprietary rights, nor should it be construed as an endorsement or recommendation on the part of the IAEA.*

*The IAEA has no responsibility for the persistence or accuracy of URLs for external or third party Internet web sites referred to in this publication and does not guarantee that any content on such web sites is, or will remain, accurate or appropriate.*

**CONTENTS**

## SUMMARY

Excessive nutrients entering water bodies – mostly originating from agricultural activities – are causing environmental challenges such as water quality degradation and eutrophication. Determining the isotopic composition of contaminants, for example nitrate ($\delta^{15}N$ and $\delta^{18}O$), allows better identification of its sources and flow paths. This in turn can assist in improving soil management practices and mitigation of contamination.

In this publication, Standard Operating Procedures (SOP) are presented on (1) methods in collection and preservation of water samples, (2) instructions on using the bacterial denitrifier and laser spectroscopy methods to determine isotopic composition of $\delta^{15}N$ and $\delta^{18}O$ of dissolved nitrate, and (3) guidance on determining enriched $\delta^{15}N$ signatures of ammonium and nitrate through micro-diffusion.

The first SOP covers how water sampling and sample preservation should be carried out in the field. Guidance is provided in ensuring that samples are representative and changes in the original isotopic signature of the contaminant due to fractionation do not occur. Avoiding fractionation is essential to ensure that identification of the source of contamination is correctly implemented. Recommendations are provided for water sampling involving sample preparation for analysis of (1) major ions in surface water, such as $Na^+$, $Mg^{2+}$, $Ca^{2+}$, $K^+$, $Sr^{2+}$, $Cl^-$, $SO_4^{2-}$, $HCO_3^-$, $NO_3^-$, $NH_4^+$ and $PO_4^{3-}$, (2) $\delta^2H$ and $\delta^{18}O$ in water, (3) $\delta^{15}N$ and $\delta^{18}O$ of nitrate in water, (4) $\delta^{34}S$ and $\delta^{18}O$ of sulphate in water and (5) $\delta^{15}N$ and $\delta^{13}C$ of total particulate organic matter in water.

The second SOP illustrates in detail how bacterial denitrification through the use of *Pseudomonas aureofaciens*, a naturally occurring bacterium, can be applied to convert nitrate in water samples into nitrous oxide ($N_2O$) and how the isotopic ratio of $\delta^{15}N$ and $\delta^{18}O$ of the produced $N_2O$ is measured by innovative laser spectroscopy analysis. The advantages of this method are the small sample volume required, that no toxic chemicals are added, and that it can be used for samples with low concentrations of nitrate. This SOP provides step-by-step instructions on (1) preparation of *Pseudomonas aureofaciens* bacterial culture for denitrification (2) conversion of $NO_3^-$ to $N_2O$ in water samples, (3) measurement of $N_2O$ using the laser spectroscopy method and (4) normalization of the data measured. It also contains a technology Annex whereby the details of a specialized purging block are shared.

The third and last SOP presents the micro-diffusion method for determining the stable nitrogen isotope composition ($^{15}N/^{14}N$) of ammonium and nitrate/nitrite in liquid samples. It provides general instructions for sample preparation, prior to analysis of $^{15}N/^{14}N$ with the isotope ratio mass spectrometry (IRMS). The ability to measure $^{15}N$ enriched nitrate and ammonium can be applied e.g. in fertilizer use efficiency studies, N-balance studies or mineralization-immobilization quantification.

# CHAPTER 1. WATER SAMPLING AND PRESERVATION FOR STABLE ISOTOPE ANALYSES OF WATER MOLECULES AND SELECTED SOLUTES

G. SKRZYPEK

## 1.1. SCOPE AND APPLICATION

The hydro- and hydrogeological processes, and the identification of pollution sources and pathways can all be studied by examining the stable isotope composition of water molecules [$\delta^2H(H_2O)$ and $\delta^{18}O(H_2O)$], the solutes in water [$\delta^{15}N(NO_3)$, $\delta^{18}O(NO_3)$, $\delta^{34}S(SO_4)$, $\delta^{18}O(SO_4)$, $\delta^{13}C(DIC)$] or the particulate organic matter [$\delta^{13}C(POM)$ and $\delta^{15}N(POM)$], together with the major ion concentration in water such as $Na^+$, $Ca^{2+}$, $Cl^-$, $SO_4^{2-}$, $NO_3^-$ and $NH_4^+$ (e.g.[1.1], [1.2]). Basic hydrochemistry methods are important for identification of pollution levels but the stable isotope methods are critical for calculation of evaporation, evapo-concentration, source identification, and for untangling water budgets from pollution budgets (e.g. [1.3]). Techniques that combine hydrochemical and stable isotope methods have therefore become essential tools for identification of non-particulate, non-source agricultural pollutants (e.g. [1.4], [1.5]). The major advantages of these combined techniques are their relatively low cost and their high success rates, even when used in areas lacking long term monitoring and baseline data.

## 1.2. PRINCIPLES

This SOP guideline provides step-by-step instructions on how to collect and preserve water samples for stable isotope analyses. The correct implementation of the SOP is essential to ensure that the initial stable isotope composition is preserved and fully reflects the composition observed under natural conditions. Adherence to the SOP helps to avoid post-collection fractionation, which may modify the original stable isotope composition. This SOP provides general guidance for water sample collection and describes specific requirements for the following analyses:

- The major ion concentrations in water ($Na^+$, $Mg^{2+}$, $Ca^{2+}$, $K^+$, $Sr^{2+}$, $Cl^-$, $SO_4^{2-}$, $HCO_3^-$, $NO_3^-$, $NH_4^+$ and $PO_4^{3-}$);
- Stable hydrogen and oxygen isotope composition of water – $\delta^2H(H_2O)$, $\delta^{18}O(H_2O)$;
- Stable nitrogen and oxygen isotope composition of nitrates in water – $\delta^{15}N(NO_3)$, $\delta^{18}O(NO_3)$;
- Stable sulphur and oxygen isotope composition of sulphates in water – $\delta^{34}S(SO_4)$ and $\delta^{18}O(SO_4)$; and stable carbon composition of dissolved inorganic carbon (DIC) in water – $\delta^{13}C(DIC)$;
- Stable nitrogen and carbon isotope composition of particulate organic matter (POM) in water samples – $\delta^{15}N(POM)$ and $\delta^{13}C(POM)$.

## 1.3. PROCEDURES

### 1.3.1. General water sampling recommendations and requirements

#### *1.3.1.1. Typical sample*

The size of water samples typically varies between 20 mL and 4 L, depending on the type and number of analyses to be performed and the analytical method available at the contracted laboratory. For some analyses, minimum volume is also required to consider sample as representative, allow safe storage and convenient handling.

#### *1.3.1.2. Materials and equipment*

Various types of vials and bottles are used for water sample collection. The choice of type and size of bottle depends on the types of analyses and the expected concentrations of the solutes of interest. Some analyses require sterile, specifically precleaned containers, whereas others need only ordinary analytical grade bottles. All containers, however, need to be clean and dry. Contamination, leaks and evaporation of the sample must be strictly avoided. Glass and high density polyethylene (HDPE) containers are the most frequently used types (see Fig.1.1.).

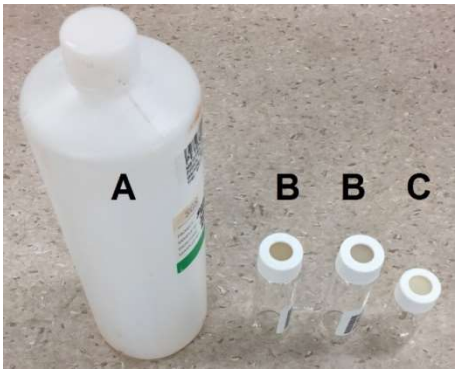



*FIG. 1.1. Example of a typical set of containers for water sampling (A) 1 $dm^3$ or 2 $dm^3$ HDPE bottle (stable isotope composition of POM, sulphates, phosphates or major ion concentrations – usually one bottle per each component to be analysed); (B) 40 mL precleaned vials with septa (stable isotope composition of nitrates or DIC – one bottle per each component); (C) 20 mL vial with septum (stable isotope composition of water; septum is not essential).*

#### *1.3.1.3. Sampling procedure*

The aim is to collect samples which are representative of the environment of interest.

For **surface water** sampling from lakes, ponds or dam reservoirs, particularly important considerations are the homogeneity of the water body, the direction of the water flow and the mixing and locations of the tributaries flowing into the water body. Large or inhomogeneous water bodies may require multiple sampling points at different locations and depths to obtain representative results. Usually, for surface water, surface “grab” samples are collected from a depth of about 10–20 cm. Locations to avoid include unusually shallow locations and those

separated from the main water body water, which are characterized by very low circulation or high evaporation of water and disruption of bottom sediments during sampling. For flowing surface waters (e.g. rivers, canals or creeks), sampling from a well-mixed central part of the main stream is recommended, if accessible and practical. For large rivers, multiple samples from cross-sections and depth profiles may be required (respectively to the research questions and required level of details).

| | |
|---|---|
| **Step 1.** Collect samples using a clean sampling HDPE container attached to a pole, which allows access to deeper water. If needed, the sample collector can stand on a walkway accessing the centre of an enclosed waterbody or a bridge. |  |
| **Step 2.** Immerse the sampling container initially in the water, keeping it bottom up. Splashing and mixing should be avoided as much as possible. |  |
| **Step 3.** Rinse the sampling container at least three times before water collection. |  |
| **Step 4.** Collect surface water from the depth of 10 – 20 cm. |  |

**Groundwater samples**, whenever available, should be collected from bores or piezometers after pumping out three volumes of the water in the bore. Bore and piezometer pumping can be conducted using an existing infrastructure or a portable submersible pump operated by a car battery (12V). Efficient bore purging requires pumping out of three water volumes of the bore (Equations 1.1 and 1.2) and measuring the pumping time (if the flow is known) or measuring the pump yield (e.g. the volume of water pumped into a bucket). Alternatively, the basic parameters of outflowing water (electric conductivity, $O_2$ concentration and pH) can be monitored using a portable multiprobe until all values are constant.

*Calculation of water volume in a bore or piezometer*
*D* – total bore depth (m), to be tested using a deeper or known from the bore construction diagram; *WT* – water table depth below ground (m) to be tested using a groundwater depth gauge; *O* – bore diameter (mm) to be measured using a ruler or determined from the bore construction diagram; *V* – calculated volume of water in the bore, in litres [$dm^3$]

Full equation:

$$V = (D - WT) \times 3.14 \times (0.5 \times 0.001 \times O)^2 \times 1000 \quad [dm^3] \qquad (1.1)$$

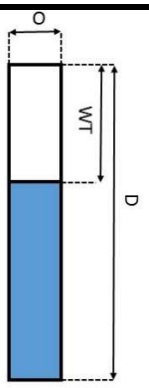
Simplified equation:

$$V = (D - WT) \times O^2 \times 0.000785 \qquad [dm^3] \qquad (1.2)$$

*Calculation example:*
Total bore depth *D* = 30m
Water table *WT* = 5m
Bore diameter *O* = 50mm (frequent size for piezometers)

The volume of this bore can be calculated as follows:
*V* = (30-5) × $50^2$ × 0.000785 and equals 49 $dm^3$.

Since purging of a minimum three bore volumes is recommended, the required pump-out is 3×49 $dm^3$ or 147 $dm^3$ (approximately 10 buckets). The average pumping speed of an electrical 12V DC submersible pump is about 5 to 15 $dm^3$/min, so approximately 10 min is required to purge a bore of this size.




Production bores operating continuously can be sampled directly from an infrastructure outlet without additional purging. Open, dug wells should be avoided if possible, because the stable isotope and hydrochemical composition of the water in the well may not reflect the water in the aquifer due to exposure to oxygen, evaporation and exchange with the atmosphere. For the same reason, sampling groundwater from bores and piezometers using a bailer is also not recommended.

*1.3.1.4. Sample storage and transportation*

Direct exposure of water samples to heat or sunlight should be avoided. During the fieldwork, the collected water samples should be stored in a dark and cold container, such as an insulated portable ice chest box (also called an esky, igloo or tourist fridge) containing ice bricks (see Fig. 1.2.). However, the ice bricks should be separated from the samples to avoid sample freezing. In the laboratory, the water samples should be stored in a fridge or cold room at approximately 4°C. **Care should be taken that samples do not freeze;** for example, because of touching the fridge walls or the cooling system.

*FIG. 1.2. Examples of insulated containers for water sample storage during fieldwork.*

**Do not freeze samples** (unless specifically recommended). During freezing, full HDPE bottles may easily lose their seal, whereas glass vials can break due to water density changes during freezing. Samples will degas and DIC will be lost.

*FIG. 1.3. Packing water samples in an ice chest box. The samples are isolated from the blue ice bricks with bubble wrap to avoid freezing and the samples are wrapped in bubble wrap to prevent cracking.*

*Precautions*

- Consider collecting duplicate samples, particularly if samples are to be shipped to overseas laboratories. Duplicates can be stored locally until the safe arrival of the shipped samples at the stable laboratory is confirmed.
- Disposable laboratory gloves should be used during sampling to avoid sample contamination and for safety reasons, particularly if a potentially hazardous water hydrochemical composition or biological contamination is a concern.

### 1.3.2. Major ion hydrochemical composition ($Na^+$, $Mg^{2+}$, $Ca^{2+}$, $K^+$, $Sr^{2+}$, $Cl^-$, $SO_4^{2-}$, $HCO_3^-$, $NO_3^-$, $NH_4^+$ and $PO_4^{3-}$)

#### *1.3.2.1. Materials and sample sizes*

Two sampling bottles – one 1 $dm^3$ HDPE plastic bottle with a cap for $Na^+$, $Mg^{2+}$, $Ca^{2+}$, $K^+$, $Sr^{2+}$, $Cl^-$, $SO_4^{2-}$ and $HCO_3^-$ and one 0.25 $dm^3$ HDPE plastic bottle with a cap for $NO_3^-$, $NH_4^+$ and $PO_4^{3-}$. The bottles need to be clean and dry, but no preservatives are required. It is highly recommended that the HDPE plastic bottles be acid pre-washed (using 4% HCl) and thoroughly rinsed with deionized water (brand new bottles of analytical grade frequently do not require additional washing).

*FIG. 1.4. Examples of sampling bottles, 1 $dm^3$ and 0.5 $dm^3$ HDPE plastic bottles.*

#### *1.3.2.2. Sampling procedure*

- Label the 1 $dm^3$ HDPE plastic bottles (Project/Location/Date; e.g. *TC0001/Danube104-01/5JAN2018*).
- Collect water sample as described in Paragraph 1.3.1.
- Open the 1 $dm^3$ HDPE plastic bottle (see Fig. 1.4.), fill it to approximately ¼ of the volume and replace the cap. Water sample bottle can be filled directly from the sampling container for surface waters or a pump outlet for groundwater, however, excessive splashing, overfilling or water aeration should be avoided as far as possible.
- Rotate and shake the bottle gently to rinse the bottle with sampling water, decant the water onto the ground – repeat this rinse three times.
- Fill the bottle with sampling water to the very top. Avoiding water splashing and forming a foam, and then replace the cap, avoiding the headspace. Do not overfill the sample container.
- Place the bottle in the ice chest box, with ice bricks at the bottom, for temporary storage during fieldwork. Upon return to the local laboratory, transfer the samples to a fridge (4°C). **Do not freeze**.
- Ship the samples to the hydrochemical analytical laboratory as soon as possible, preferably within 48 h.

*For nitrogen and phosphorous concentration analyses, follow the procedure as above; however, use additional 0.25 $dm^3$ bottles and add approximately 2 mL of $H_2SO_4$ to lower the pH to below pH 2. Bottles with $H_2SO_4$ acid can be prepared in the laboratory prior the fieldwork but should be clearly labelled and they cannot be rinsed with samples in the field (this would washout acid from bottles).*

### 1.3.3. Stable hydrogen and oxygen isotope composition of water – $\delta^2H(H_2O)$ and $\delta^{18}O(H_2O)$

*1.3.3.1. Materials and sample size*

This analysis does not require a special type of vial. The use of 20 to 50 mL glass vials with septa is recommended to ensure a very good seal and prevent leakage and evaporation. The vials do not require special pretreatment or cleaning; however, they need to be clean and dry. Here (Fig. 1.5.), Thermo Scientific 20 mL EPA vials are given as an example (p/n B7920, origin USA).

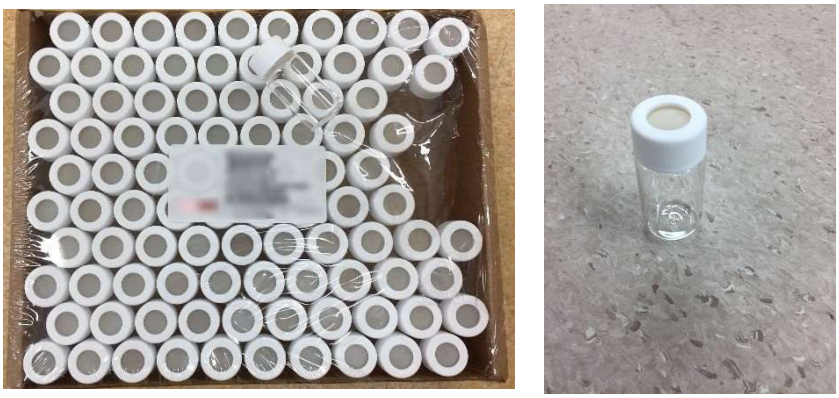


*FIG. 1.5. Example of 20mL vials for water sampling.*

*1.3.3.2. Sampling procedure*

- Label the 20 mL vial (e.g. Project/Location-bottle/Date; *TC5501/Danube104-01/5JAN2018*).
- Collect water sample as described in Paragraph 1.3.1.
- Open the 20 mL vial (see Fig. 1.5.), fill it with approximately ¼ of the volume and replace the cap.
- Rotate and shake the vial gently to rinse the vial and the cap with sampling water. Decant the water – repeat this rinsing three times.
- Fill up the bottle with a water sample to the rim. Avoid water splashing and forming a foam. Replace the cap firmly, avoiding the headspace.
- Place the bottle in the ice chest box (ice bricks are not essential here) for temporary storage during fieldwork and then transfer to a fridge (4°C) upon return to the local laboratory. **Do not freeze.**
- Samples can be stored in the fridge for a few weeks before shipping to the stable isotope laboratory.

### 1.3.4. Stable nitrogen and oxygen isotope composition of nitrates in water, $\delta^{15}N(NO_3)$ and $\delta^{18}O(NO_3)$, and stable carbon isotope composition of DIC in water, $\delta^{13}C(DIC)$

Materials and the procedure are the same for both the stable nitrogen and oxygen isotope composition of nitrates and the stable carbon isotope analyses of DIC.

*1.3.4.1. Materials and sample size*

- Sampling vial – 40 mL. This analysis requires precleaned glass vials, such as e.g. Thermo Scientific 40 mL Vials, Certified 300 Series (p/n S336-0040, origin USA).
- 50 mL disposable syringes
- Sterile 0.21 µm pore size and 25 mm diameter syringe filter discs (e.g. Pall Corporation p/n 4612).


*FIG. 1.6. Examples of 40 mL vials, 50 mL disposable syringes and sterile 0.21µm syringe filters.*

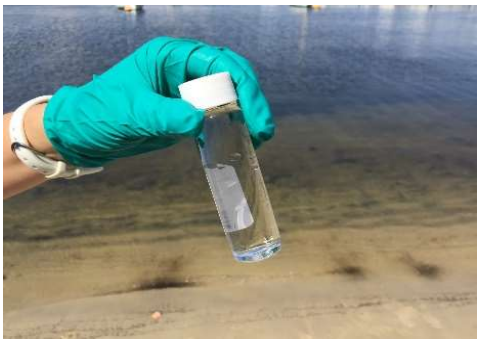
*1.3.4.2.Sampling procedure*

Note that this procedure uses filtration as the method of sample sterilization. Do not use methods involving sample poisoning with toxic chemicals for sample sterilization (e.g. mercury chloride, ethanol or chloroform). Samples sterilized using toxic chemicals cannot be analysed for their stable isotope compositions using the bacterial denitrification method.

| | |
|---|---|
| **Step 1.** Label the 20 mL vials with Project/Location/Date | *e.g. TC5501/Danube104-1/5JAN2018* |
| **Step 2.** Collect a water sample in a sampling container | *Follow instructions from Paragraph 1.3.1* |
| **Step 3.** Fill a 50 mL syringe with a water sample, remove any excessive headspace (a small bubble is OK). Make sure the syringe is very full, as approximately 50 mL of water will be needed (approximately 10 mL for rinsing and approximately 40 mL for filling the vial). |  |

| | |
|---|---|
| **Step 4.** Attach the 0.21 µm syringe filter to the syringe full of water, making sure it is firmly attached (some syringes have threads and the filter can be screwed on securely). The filter is sterile, so do not touch the blue part, particularly the outlet tip. If the amount of water in the syringe is insufficient, the filter can be detached and carefully placed in the original sterile package with the blue part down. The syringe can be refilled, the filter reattached and the filtration continued.<br><br>If for any reason the filter becomes contaminated (e.g. dropped on the ground, the tip touched by hand or wetted with raw unfiltered sample) discard the filter and replace it with a new one.<br><br>If the samples contain a lot of suspensions, 2 or 3 filters may be required. For extremely dirty water, an initial filtration using a 0.45 µm syringe filter may be required. |  <br>  |
| **Step 5.** Open a 40 mL precleaned vial and carefully remove the cap with the septum.<br><br>Do not touch the internal part or the rim of the vial or the internal part of the cap. |  <br>  |

| | |
|---|---|
| **Step 6.** First, the vial needs to be rinsed with filtered water sample. Filter water directly into the vial by pushing down on the plunger of the syringe. Avoid splashing unfiltered water. Fill the vial about ¼ full with filtered water, replace the cap and rinse the vial once by gently shaking and rotating the vial. Open the vial and decant the water. |  <br>  |
| **Step 7.** Fill up the vial with filtered water to the rim and fit the cap firmly, avoiding the headspace.<br><br>Note that pushing the syringe plunger may require more force as the filtration progresses and more suspension plugs the filter. Usually, one filter is sufficient to filter 50 mL of water; however, if necessary, replace the filter and continue the filtration until the vial is filled. |  <br>  |

| | |
|---|---|
| **Step 8.** Store the vial with its water sample in the ice chest box on ice bricks during the fieldwork. Make sure that the water sample does not freeze. Cover the ice bricks with bubble wrap foil or a towel. |  |
| **Step 9.** Upon return to the local laboratory, transfer the vials to the fridge (4°C) for storage until shipping them to the stable isotope laboratory. Make sure that the vials are not touching the walls of the fridge and will not freeze. The filtered samples can be stored in the fridge for few weeks. For shipping of samples use an ice chest box with ice bricks and secure the samples using bubble wrap. Use a door-to-door 48 h or 72 h courier service. |  |

### 1.3.5. Stable sulphur and oxygen isotope composition of sulphates in water, $\delta^{34}S(SO_4)$ and $\delta^{18}O(SO_4)$

The storage, of unfiltered water samples for an extended period of time, even in a fridge at 4°C, may cause an unwanted stable isotope fractionation which may compromise the results. Therefore, conducting the sample filtration and precipitation of barium sulphate ($BaSO_4$) is recommended as soon as practicable, preferably within 2-3 days of the sample collection.

Knowing the approximate $SO_4^{2-}$ concentration is useful when calculating the required sample volume and amount of reagents required for complete precipitation of $SO_4^{2-}$ in the form of $BaSO_4$.

#### *1.3.5.1. Sample collection and sample size calculation*

The sampling procedure is the same as for major ion concentrations; see Paragraph 1.3.2. The only differences are:

- The size of the HDPE sample plastic bottle may vary between 0.5 and 4 $dm^3$. The sample volume needs to be estimated based on $SO_4^{2-}$ concentration in the water samples.
- Water samples should not be shipped to the stable isotope laboratory. $BaSO_4$ from water samples should be precipitated locally and only dry $BaSO_4$ powder should be shipped to the stable isotope laboratory. $BaSO_4$ is chemically stable and can be stored at room temperature for many years.

*Calculation of sample volume*

A minimum of 2 mg of dry $BaSO_4$ is required for the stable sulphur and oxygen isotope analyses. However, this small amount is difficult to handle during the analytical procedure and easily can be lost during a washing procedure. Therefore, precipitation of minimum 50 mg (or more e.g. 100-200 mg) of $BaSO_4$ is recommended. Following the atomic masses and reaction of barium sulphate precipitation, $SO_4^{2-}(aq) + BaCl_2\ (aq) \rightarrow BaSO_4\ (solid)$, 1 mg of $SO_4^{2-}$ dissolved in water will form 2.4 mg of $BaSO_4$, this will require 2.2 mg of $BaCl_2$. Therefore, in order to precipitate the required 50 mg of $BaSO_4$, 20.6 mg of $SO_4^{2-}$ in water sample is needed and it needs to be reacted with 44.4 mg of $BaCl_2$ what equals ~0.4 mL of 10% $BaCl_2$ in water solution. As a precaution 3 times more $BaCl_2$ should be used to make sure that all $SO_4^{2-}$ is completely and quickly precipitated. Therefore, about 1.2 mL of 10% $BaCl_2$ in water solution needs to be added to a volume of water sample containing 20.6 mg of $SO_4^{2-}$.

If the $SO_4^{2-}$ concentration ($C_{SO4}$) in water is known (mg/dm$^3$), the required volume ($V_S$) of the sample (dm$^3$) for 50 mg of $BaSO_4$ precipitation can be calculated as follows: $20.60/C_{SO4} = V_S$.

*Calculation examples:*
$V_s$ – volume required for precipitation of 50 mg of $BaSO_4$

$C_{SO4}$ – $SO_4^{2-}$ concentration in water is 50 mg/dm$^3$
$V_s = 20.6/50 = 0.4$ dm$^3$

$C_{SO4}$ – $SO_4^{2-}$ concentration in water is 5 mg/dm$^3$
$V_s = 20.6/5 = 4.1$ dm$^3$

If the $SO_4^{2-}$ concentration ($C_{SO4}$) in water is unknown (mg/dm$^3$), it can be roughly estimated based on the electrical conductivity (*EC*) measured, using a portable EC probe and assuming that $SO_4^{2-}$ concentration accounts for approximately 10–20% of the total mass of solutes (this is a common range for fresh waters).

If the EC (*EC*) in water is known (µS), the required volume ($V_S$) of the sample (dm$^3$) can be calculated as follows: $20.6 / (EC \times 0.65 \times 0.1) = V_S$.

*Calculation examples:*
$V_s$ – volume required for precipitation of 50 mg of $BaSO_4$

*EC of* water is 500 µS
$V_s = 20.6/ (500 \times 0.65 \times 0.1) = 0.6$ dm$^3$

*EC of* water is 60 µS

$V_s$ = 20.6/ (60×0.65×0.1) = 5.3 $dm^3$

### *1.3.5.2. Materials and equipment*

*Chemicals*

Read all safety information sheets for the chemicals listed below before proceeding with the precipitation procedure. Barium chloride in powder or crystalline form is **hazardous and toxic** if swollen or inhaled. Use appropriate protection: gloves, goggles and face mask. The solution is not hazardous due to its low concentration, but it can be harmful if swallowed. $BaCl_2$ is also toxic to aquatic wildlife; therefore, all waste needs to be treated before discharging down the sink.

- Barium chloride ~10% solution 500mL, add 55 g $BaCl_2 \cdot 2H_2O$ to 455 mL of deionized water;
- ~5N HCl analytical grade 500mL, add 205 mL (37%) HCl to 295 mL deionized water (always add acid to water not the other way around);
- 3 to 10% $H_2O_2$ 500 mL (optional for organic matter removal);
- ~5% zinc acetate solution 500 mL (optional for $H_2S$ removal), add 25 g zinc acetate to 475 mL deionized water;

*For waste neutralization only*

- ~5N $H_2SO_4$ 500 mL, mix 70 mL of highly concentrated $H_2SO_4$ (98%) with 430 mL deionized water (always add acid to water not the other way around);
- Soda ash – technical grade;

If different concentrations of chemicals will be used, the calculations need to be adjusted.

*Equipment*

- Filtration system and 0.45 µm filter papers;
- 500 and 1000 mL beakers;
- Evaporating dishes (50 mL) or beakers (50 mL);
- Hotplate or hot water bath (40 °C);
- Stirrers and metal spatula;
- pH strips (pH 0–7);
- Siphon = glass tubing (approx. 25 cm in length) with 50 cm plastic tubing attached;
- Waste collection vessel – e.g. plastic bucket approximately 10-15 $dm^3$

All glassware needs to be washed and rinsed with deionized water and dried.

### *1.3.5.3. Precipitation procedure*

| | |
|---|---|
| **Step 1 (optional)**<br>If the water sample contains dissolved $H_2S$ and its concentration is higher than<br>0.5 mg/dm$^3$ (a distinctive rotten-egg odour), it should be precipitated first to separate it from $SO_4^{2-}$:<br><br>transfer the water sample to a glass beaker (500 or 1000 mL depending on the sample volume), add 5% zinc acetate solution in a 1:10 ratio (e.g. 50 mL zinc acetate solution to 500 mL of water sample volume), mix it very well and let it stand for 1 h. |  |
| **Step 2**<br>Filter the sample using a filtration system and 0.45 µm filter paper.<br><br>Various systems are available and can be attached to a suction/vacuum line or to a portable peristaltic pump. |  <br>   |
| **Step 3**<br>Transfer the sample to a beaker and adjust pH to 2– 3 by adding approximately 1–3 mL of ~5N HCl and stirring it with a glass rod. Warm up the solution to ~40 °C on a hotplate. If any precipitation occurs, e.g. organic matter coagulation, the water sample needs to be refiltered. |  |

**Step 4**

Remove the beaker from the hotplate and add 10% $BaCl_2$ solution. The volume of the required $BaCl_2$ solution depends on the sample volume and $SO_4^{2-}$ concentration. Approximately 1.2 mL should be added to completely precipitate 50 mg of $BaSO_4$ (as a precaution it is three times more than required). Stir the sample for approximately 1 minute using a glass or ceramic stirring rod and firmly touching the wall of the beaker to trigger precipitation. For low concentrations, the precipitation will not start immediately and may start within couple of hours. Cover the sample with aluminium foil or e.g. parafilm and leave it overnight.

**Step 5**

Add a single drop of 10% $BaCl_2$ solution to each beaker to test if any further precipitation occurs. If it does, a cloudy trace will develop – these samples will require additional $BaCl_2$ solution to fully precipitate the $SO_4^{2-}$.

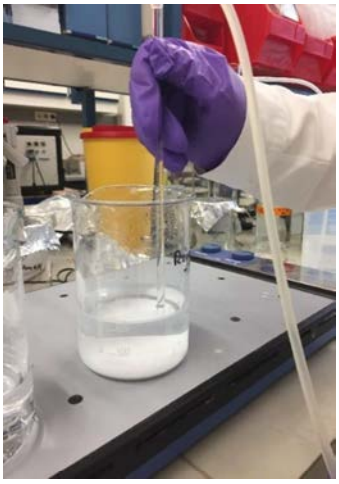
If precipitation is completed, carefully remove the water using a siphon, leaving a minimum amount of water. Be careful not to suck into the siphon and discharge the $BaSO_4$ from the bottom of the beaker. If required, pipette out the water from the bottom of the beaker (the less water left = more efficient washing).

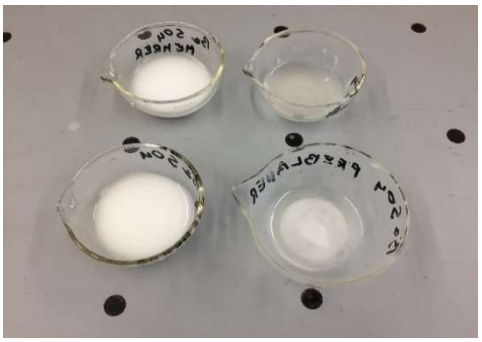
Collect all wastewater in a bucket for later neutralization.

Wash the siphon thoroughly with deionized water between samples and avoid contact with the discharged water rinsing with deionized water from outside and pumping some deionized water trough.

After removing the water, wash the precipitate by filling the beaker with deionized water, stir well and leave it to settle for approximately 5 h. Repeat this washing and settling three times.

If a laboratory centrifuge is available, the washing procedure can be speeded up by transferring samples to 50 mL centrifuge tubes and spinning them at ~5000 rpm to accelerate $BaSO_4$ settling. Decant the water, refill with deionized water and spin the sample again –repeat four times.



| | |
|---|---|
| **Step 6**<br>After the final wash, add a small amount of water (10–20 mL) and swirl to loosen the precipitate. Pour it into an evaporating dish. Use deionized water to remove any remaining precipitate and transfer it to a small 50 mL evaporation dish or beaker. Dry in an oven at 110 °C overnight.<br><br>Pure $BaSO_4$ is white. The brown coloration on the photo results from a very high concentration of dissolved organic matter (DOM). If present, DOM needs to be removed. |  <br>  |
| **Step 7 (optional)**<br>Remove the dry sample from the oven and add 3-10% $H_2O_2$ to cover the sample with 1–2cm of liquid, stir and leave to react overnight. Repeat this step if any brown coloration remains.<br><br>Place the precipitate sample in an oven at 110 °C again and dry overnight.<br><br>Alternatively, DOM can be removed by baking the dry $BaSO_4$ in a furnace at 500 °C overnight. For this procedure, the samples need to be transferred to a ceramic pre-baked crucible. |  |

| | |
|---|---|
| **Step 8**<br>Transfer dry $BaSO_4$ using a spatula to small 1.5 mL vials or hand-made aluminium envelopes and place them separately in small Ziploc bags. |  <br>  |
| **Step 9**<br>Attention: Unreacted $BaCl_2$ from samples can be harmful to aquatic life and cannot be discarded down the sink.<br><br>For waste neutralization, add approximately 5 mL ~5N $H_2SO_4$ per 1 $dm^3$ of waste to precipitate the remaining barium chloride in the form of $BaSO_4$ (non-hazardous). Leave it overnight, and then neutralize the acidity with soda ash to a pH of approximately 6–7 and pour the waste down the sink. | $BaCl_2$<br>Toxic if swallowed<br>Harmful if inhaled<br><br>  <br><br>For further information use safety sheets (MSDS) from the chemical supplier (e.g.http://www.sciencelab.com/msds.php?msdsId=9927447) |

### 1.3.6. Stable nitrogen and carbon isotope composition of total particulate organic matter in water (POM), $\delta^{15}N$ (solids) and $\delta^{13}C$ (solids)

The water samples need to be filtered through glass microfiber filters to collect suspensions. Only organic matter on the filters will be analysed; therefore, the filtered water can be discarded. If a carbonate suspension is expected in the water samples, the filters need to be acidified to remove carbonates prior the stable carbon isotope analyses. However, for the stable nitrogen analyses, the filters cannot be acidified; therefore, if both nitrogen and carbon need to be analysed, two samples need to be prepared separately (one for N and one for C).

#### *1.3.6.1. Sample collection*

The sampling procedure is similar to the one described for major ion concentrations (see Paragraph 1.3.2.). The only differences are:

- While collecting water samples, take care, avoid floating debris and disturbing the sediments at the bottom of the lake or river.
- The size of the HDPE sample plastic bottle may vary, usually between 0.5 and 4 $dm^3$, depending on the concentration of the suspension.
- Water samples can be filtered in the field to reduce the amount of water transported to the laboratory or, if more practical, the samples can be filtered after returning to the laboratory within 3 days of sampling. Prior to filtration, the water samples should be kept in a fridge at 4 °C.

#### *1.3.6.2. Materials and equipment*

- Filtration system and 0.45 µm pre-baked glass microfiber (e.g. Whatman Binder-Free Glass Microfiber Filters) filter papers, preferably 25 or 45 mm diameter. The filter papers need to be prepared prior to the filtration by prebaking in a furnace overnight at 500°C.
- Stainless steel forceps and spatula for handling filter papers.

#### *1.3.6.3. Procedure*

| | |
|---|---|
| Filter samples using a filtration system and 0.45 µm glass microfiber filter papers (GF/F).<br><br>The filters should be handled with care, using forceps when placing them into the filtration system. The filtration system should be washed/rinsed with deionized water between samples.<br><br>Various filtration systems are available and can be attached to a suction/vacuum line if used in the laboratory or to a portable peristaltic pump if used in the field (as in the photo). |  |

| | |
|---|---|
| The amount of filtered water will vary and it is difficult to estimate beforehand because of the variable amount of the suspension in water and the variable nitrogen and carbon concentrations in the suspension. A rule of thumb is to filter until the filter is completely clogged and filtration becomes very slow (~0.01 mg of N and ~0.10 mg of C is required for the stable isotope analysis). Record the amount of filtered water for later calculation of the concentration of N and C per volume (mg/dm$^3$). |  |
| Remove the filter from the filtration system and fold it in half using the spatula and forceps, this will help to keeping the suspension inside. Place it on a petri dish or on a piece of aluminium foil and dry in an oven overnight at 50–60 °C.<br><br>If filtered in the field, fold filter papers in half and pack in aluminium foil envelope and small Ziploc bags (each separately). Avoid touching with foil the part of filter covered with sample. Place the Ziploc bags in a plastic box and then in the ice chest box with ice bricks. Upon returning to the local laboratory, the samples on filters can be stored in a fridge at 4 °C for a few days, or frozen at -20 °C for several months. The samples need to be dried before shipping to the stable isotope laboratory. |  <br>  <br>  |
| If a carbonate suspension is expected in the water samples, two glass filter papers need to be prepared separately. One for $\delta^{15}N$ one for $\delta^{13}C$ analyses as described above, the filter for $\delta^{13}C$ analyses will require an additional acidification step, to be performed in the stable isotope laboratory. |  |

## REFERENCES TO CHAPTER 1


[1.1] MICHENER, R., LAJTHA, K., Stable Isotopes in Ecology and Environmental Science. John Wiley & Sons (2008) 594.

[1.2] SKRZYPEK, G., DOGRAMACI, S., GRIERSON, P.F., Geochemical and hydrological processes controlling groundwater salinity of a large inland wetland of north-west Australia. Chemical Geology **357** (2013) 164–177.

[1.3] KENDALL, C., DONNELL, J.J., Isotope Tracers in Catchment Hydrology. Elsevier Science (1999) 839.

[1.4] YUE, F.J., LI, S.L., LIU, C.Q., ZHAO, Z.Q., DING, H., Tracing nitrate sources with dual isotopes and long term monitoring of nitrogen species in the Yellow River, China. Scientific Reports **7** (1) (2017) 11.

[1.5] SZYNKIEWICZ, A., BORROK, D.M., GANJEGUNTE, G.K., SKRZYPEK, G., MA, L., REARICK, M., PERKINS, G., Isotopic studies of the Upper and Middle Rio Grande. Part 2 - Salt loads and human impacts in south New Mexico and west Texas. Chem. Geol. **411** (2015) 336-350.


## BIBLIOGRAPHY TO CHAPTER 1


CARMODY, R.W., PLUMMER, L.N., BUSENBERG, E., COPLEN, T.B, Methods for collection of dissolved sulfate and sulfide and analysis of their sulfur isotopic composition. USGS, Open-File Report (1998) 97-234.

U.S. Geological Survey, National field manual for the collection of water quality data: U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, chaps. A1-A10, available on-line at http://pubs.water.usgs.gov/twri9A (accessed 4/01/2018).

# CHAPTER 2. $^{15}N$ AND $^{18}O$ ISOTOPIC ANALYSIS OF NITRATE USING LASER SPECTROSCOPY AND A BACTERIAL DENITRIFIER METHOD

A. LEE ZHI YI, T.Z. YAN, M. HEILING, C. RESCH, A. TOLOZA, G. WELTIN

## 2.1. SCOPE AND FIELD OF APPLICATION

Nitrate pollution sources in water can be discriminated through the analysis of its stable $\delta^{15}N$ and $\delta^{18}O$ isotopes. *Pseudomonas aureofaciens* bacteria enzymatically convert aqueous $NO_3^-$ to gaseous $N_2O$ in a process called denitrification. The gaseous $N_2O$ can then be measured on an isotopic laser spectroscope to attain concentration, as well as signatures of $\delta^{15}N$ and $\delta^{18}O$ isotopes, which can then be utilized as tracers to understand the sources of dissolved nitrate.

The advantages of the denitrifier method compared to existing nitrate isotope discrimination methods are the small sample volume required, that no toxic chemicals are added, and that it can be used for samples with low concentrations. In addition to providing robust analysis, the laser spectroscopy method can be customized to accommodate a large sample input. Furthermore, this method is applicable in both, freshwater and seawater, and can be easily applied to any water sample sources with a nitrate contamination concentration of 2ppm and above.

This SOP was developed to provide illustrated guidance for determining the $\delta^{15}N$ and $\delta^{18}O$ isotopic composition of nitrate ($NO_3^-$) in water samples. It provides step-by-step guidance to scientists, technicians and students implementing procedures and tools to prepare samples for isotope analysis. Determination of the stable nitrogen isotope composition allows Member States to better determine and understand the sources and forms of nitrate for improved water and nutrient management practices.

## 2.2. PRINCIPLE AND EXPERIMENTAL OVERVIEW

The distinct signatures of each nitrate source (fertilizers, wastewater, manure, or organic matter) has been extensively studied [2.1, 2.2, 2.3] and is often used to trace the fate of dissolved nitrate. *Preudomonas aureofaciens,* utilized in the denitrifier method [2.4, 2.5], is a naturally occurring bacteria that lacks an $N_2O$ reductase enzyme. While it is able to convert nitrate ($NO_3^-$) to nitrous oxide ($N_2O$), the further process of conversion to nitrogen ($N_2$) is inhibited due to the lack of the reductase enzyme.

The pathway of this process is shown below:

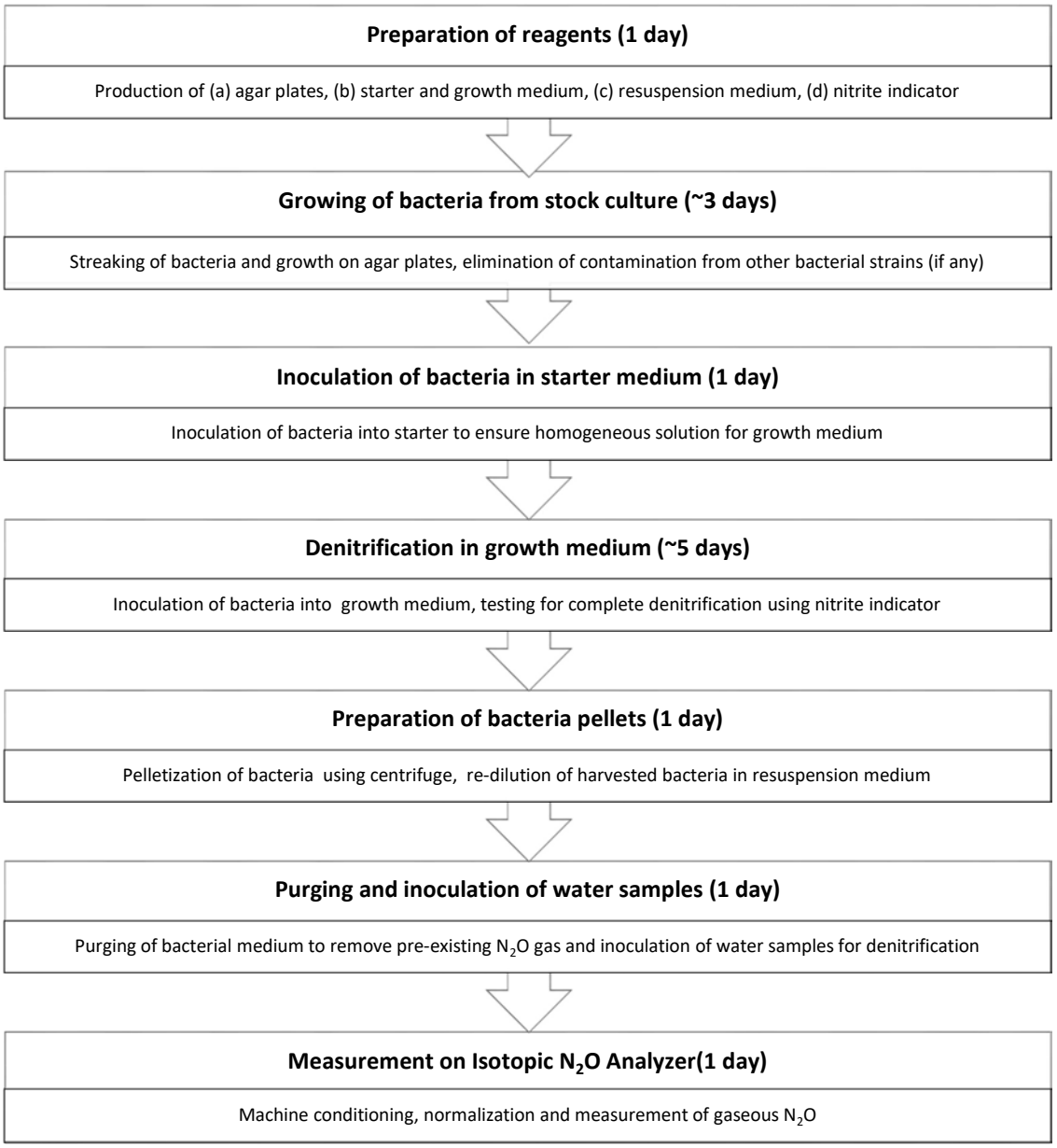
$$2NO_3^- \rightarrow 2NO_2^- \rightarrow 2NO \rightarrow N_2O \not\rightarrow N_2$$

Preliminary sample preparations are required to convert the aqueous $NO_3^-$ to gaseous $N_2O$ for measurement. The concentrated *Pseudomonas aureofaciens* bacteria are first purged with $N_2$ to create anaerobic conditions and to remove any ambient $N_2O$ which would adulterate the measurements. Then, water samples are injected and left to incubate overnight to allow for the

*Pseudomonas aureofaciens* bacteria to denitrify. The gaseous $N_2O$ is then measured on the laser spectroscope.

Prior to sample analyses the analyser is conditioned with a gas standard with an $N_2O$ concentration similar to the sample to ensure that it is ready for measurements. The raw delta values obtained from the analyser software require normalization to the international scale. For this procedure the international reference materials and in-house laboratory reference materials have to be processed the same way as all samples.

The experimental overview below describes (1) the pre-preparation process of *Pseudomonas aureofaciens* bacteria, (2) the denitrification of the water sample, and (3) the usage of a laser spectroscope.



## 2.3. PREPARATION OF BACTERIAL CULTURE FOR DENITRIFICATION

The method described below is sufficient for producing 240 mL concentrated *Pseudomonas aureofaciens* bacteria. All glassware should be washed with lab-grade detergent and dried prior to usage. Some processes should be performed in a laminar flow hood. Due to work with caustic reagents, perform steps in relevant lab protective gear.

### 2.3.1. Equipment

**Labware**

- Schott Duran bottles
  - 6 × 500 mL (wide-mouthed, cap with septa);
  - 1 × 500 mL (narrow mouthed);
  - 8 × 100 mL;
  - 2 × 50 mL
- Erlenmeyer flask
  - 1 × 3000 mL;
  - 1 × 1000 mL
- Volumetric flask
  - 2 × 50 mL
- Suba-Seal rubber septa
  - 1 × 17.5 mm (#33);
  - 1 × 24.0 mm (#49)
- Sterile 750 mL centrifuge tub (4, autoclaved);
- Sterile needles and syringes (any volume between 5-20 mL for syringe);
- Clean petri dishes (~20, 100 mm×15 mm);
- Funnel;
- Inoculating loop

**Reagents**

- Tryptic Soy Agar (TSA);
- Tryptic Soy Broth (TSB);
- Potassium nitrate ($KNO_3$);
- Ammonium chloride ($NH_4Cl$);
- Monopotassium phosphate ($KH_2PO_4$);
- Sulfanilamide ($C_6H_8N_2O_2S$);
- Phosphoric acid ($H_3PO_4$);
- N-(1-Naphthyl) ethylenediamine dihydrochloride (NED) ($C_{12}H_{14}N_2 \times 2HCl$);
- Sodium hydroxide (NaOH);
- Hydrochloric acid (HCl);
- Ethanol ($C_2H_6O$);
- Deionized Water

**Equipment**

- pH meter;
- Refrigerator (4°C) and optional: deep freezer (-70 °C);
- Incubator (~26-28 °C);
- Orbital shaker;
- Balance (± 0.001 g);
- Bunsen burner;
- Laminar flow hood

### 2.3.2. Preparation of reagents

**(a) Preparation of Agar Plates**

Prepare washed and dried clean glassware as below:

- 1 × 500 mL Erlenmeyer flask

In flask, weigh out and mix:

- 500 mL deionized water;
- 23 g Tryptic Soy Agar (TSA);
- 0.5 g potassium nitrate ($KNO_3$)

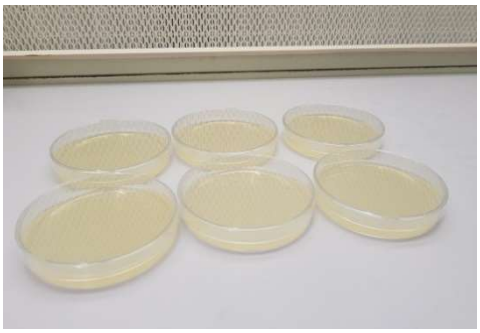
Use pH meter to check medium, pH should be 6.8±0.2. If necessary, adjust with 1M HCl or 1M NaOH

Cover flask with aluminium foil, autoclave at 120°C for 30 min.

Leave TSA media to cool for ~20 mins, or until bottle is cool enough to be handled. Do not cool for too long as media may solidify in flask.

Work in the laminar flow hood:

Ensure a homogeneous TSA media by swirling the flask carefully. Pour media into plate until it fills approximately half of the petri dish. Cover immediately to avoid contamination and leave to solidify in laminar hood. Place solidified agar plates in plastic bag and seal with tape. Label bag with type: "TSA + $NO_3^-$", date of production, name of technician. Store agar plates in cool room or refrigerator until use (4 °C).



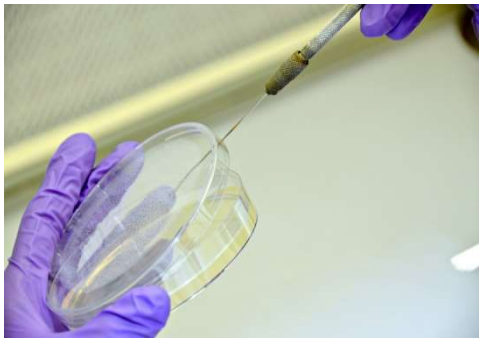
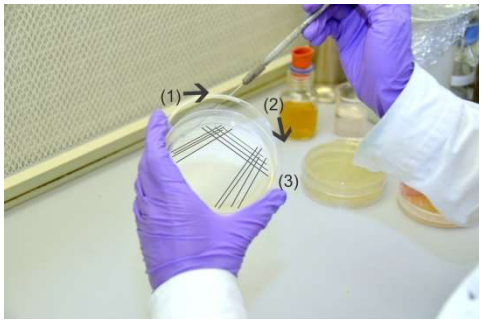
| **(b) Creation of starter and growth medium** | |
|---|---|
| Prepare washed and dried clean glassware as below:<br><br>• 6 × 500 mL Schott Duran bottles (for Growth medium);<br>• 2 × 50 mL Schott Duran bottles (for Starter medium)<br><br>In 3000 mL Erlenmeyer flask, weigh out and mix:<br><br>• 2500 mL deionized water;<br>• 75 g Tryptic Soy Broth (TSB);<br>• 2.5 g potassium nitrate ($KNO_3$);<br>• 1.25 g ammonium chloride ($NH_4Cl$);<br>• 12.25 g monopotassium phosphate ($KH_2PO_4$)<br><br>Set aside some deionized water to rinse reagents from funnel and glassware. |  <br>  |
| Use pH meter to check medium pH, should be 6.8±0.2. If necessary, adjust with 1M HCl or 1M NaOH.<br><br>Divide broth into the volumes below:<br><br>• 6 × 400 mL in 500 mL Schott Duran bottles;<br>• 2 × 40 mL in 50 mL Schott Duran bottles<br><br>Cap bottles partially to avoid overpressure in the autoclave. |  |
| Affix autoclave tape on caps and label bottle with type: "Starter, $NO_3^-$(+)" or "Growth, $NO_3^-$(+)", date of production, name of technician.<br><br>Autoclave at 120 °C for 30 min. and cap fully after. Cooled growth medium can be stored in the cold room or refrigerator up to a year. However, it is recommended to create fresh batches periodically and use as soon as possible. |  |

| **(c) Creation of resuspension medium** | |
|---|---|
| Prepare washed and dried clean glassware as below:<br><br>• 8 × 100 mL Schott Duran medium bottles (for Resuspension medium)<br><br>In a clean 1000 mL Erlenmeyer flask, weigh out and mix:<br><br>• 500 mL deionized water;<br>• 7.5 g Tryptic Soy Broth (TSB);<br>• 0.13 g ammonium chloride ($NH_4Cl$);<br>• 1.2 g monopotassium phosphate ($KH_2PO_4$)<br><br>Set aside some deionized water to rinse reagents from funnel and glassware. |  <br><br>  |
| After reagents are dissolved, check medium pH, should be 6.8±0.2. If necessary, adjust with 1M HCl or 1M NaOH.<br><br>Divide broth into the volumes below:<br><br>• 8 × 60 mL in 100 mL Schott Duran bottles<br><br>Cap bottles partially to avoid overpressure in the autoclave. |  |
| Affix autoclave tape on caps and label bottle with type: "Resuspension, $NO_3^-$(-)", date of production, name of technician.<br><br>Autoclave at 120 °C for 30 min. After medium is cooled, it can be stored in the cold room or refrigerator up to a year. However, it is recommended to create fresh batches periodically and use as soon as possible. |  |

| **(d) Creation of nitrite/nitrate indicator** | |
|---|---|
| To create Sulfanilamide solution:<br><br>In a clean 50 mL flask, weigh out and mix:<br><br>• 30 mL deionized water;<br>• 4 g Sulfanilamide ($C_6H_8N_2O_2S$);<br>• 10 mL phosphoric acid ($H_3PO_4$, 85% aqueous)<br><br>To create NED (N-(1-Naphthyl) ethylenediamine) solution:<br><br>In a clean 50 mL flask, weigh out and mix:<br><br>• 50 mL deionized water;<br>• 0.2 g NED ($C_{12}H_{14}N_2$) |  <br>  |
| Shake flasks until reagents are dissolved. Transfer solution to capped bottles; the NED solution should be stored in an amber/dark reagent bottle. Label with "Sulfanilamide" or "NED", date of production and name of technician. |  |

### 2.3.3. Procedure

| **Growing of bacteria on agar plates (Duration: 3-4 days)** | |
|---|---|
| Prepare as below and work in laminar flow hood:<br><br>• 3 × Agar plates, left to warm to room temperature.<br>• Frozen stock of *Pseudomonas aureofaciens* from the -70 °C freezer. **Do not wait for the stock to thaw!** |  |

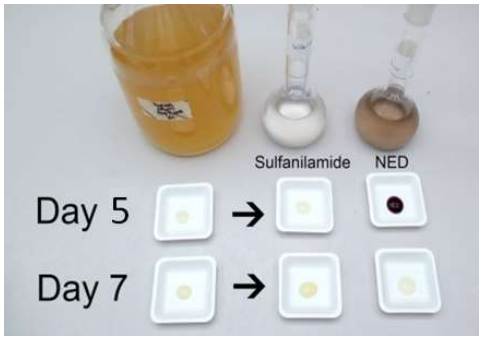
| | |
|---|---|
| Sterilize the inoculation loop until it glows red. Hold to cool for a few seconds. Do not allow contact of loop with workbench surface. |  |
| Dip inoculation loop into frozen stock to acquire bacterial slurry. Cap and return to freezer immediately after to avoid thawing. |  |
| Inoculate the bacteria onto the agar plate by gently dragging the inoculation loop across the surface, drawing four parallel lines. |  |
| Re-sterilize the inoculation loop in the flame, rotate the petri dish 90°, and draw another four parallel lines using bacteria streaked in first quadrant.<br><br>Repeat this step, careful to not touch the previously streaked sectors. At the end, the agar plate should have three sets of parallel lines. The purpose of streaking in this pattern is to "dilute" the bacteria to gain isolated colonies. |  |

| | |
|---|---|
| Cover petri dish and seal with parafilm. Label dish along the rim with strain name, plate number, date and source.<br><br>Place in incubator at 26 °C. The bacteria are orange in colour and should be ready in two days. |  |

| **Inoculation of bacteria in starter medium (Duration: 1-2 days)** | |
|---|---|
| Prepare as below and work in laminar flow hood:<br><br>• 1 × 50 mL bottle of starter medium left to warm to room temperature;<br>• 1×#33 Suba-Seal soaked overnight in 70% ethanol, 30% deionized water;<br>• *Pseudomonas aureofaciens* bacteria culture. |  |
| Briefly flame lips of growth medium bottle after opening. |  |
| Using flamed inoculation loop, extract a single colony of bacteria from agar plate. |  |

| | |
|---|---|
| Swish inoculation loop with bacteria into starter medium.<br><br>Cap immediately with rubber Suba-Seal. |  |
| Place on shaker (150 rpm) to incubate for 2 days at room temperature. When starter medium looks cloudy, it is ready for inoculation into growth medium. |  |

| **Inoculation of bacteria in growth medium (Duration: 5-7 days)** | |
|---|---|
| Prepare as below:<br><br>• 6 × 500 mL bottles of growth medium left to warm to room temperature;<br>• Sterile syringe (~5 mL) and needles. |  |
| Ensure seals are tight by screwing bottles firmly. It is important for the medium bottles to be airtight as the bacteria require an anaerobic environment to completely denitrify. |  |

| | |
|---|---|
| Using sterile needle and syringe, extract 5 mL starter solution and inject into each growth medium bottle.<br><br>Place on shaker (150 rpm) and allow bacteria to incubate for 5-7 days. |  |
| Check for denitrification from day 5 onwards. Using sterile needle and syringe, extract 1 mL of solution from the growth media bottles and place on test plates.<br><br>Using a pipette, first add 40 µL of Sulfanilamide solution. Then, add 40 µL of the NED solution. It will turn magenta if there is still $NO_2^-$/$NO_3^-$ in the medium. Continue testing every day until solution no longer turns magenta. This suggests that bacteria have completely converted $NO_3^-$ to $N_2O$ and are now ready to concentrate. |  |
| This bacterial solution can be used as a starter for subsequent batches:<br><br>Transfer 5 mL of bacteria suspension from the denitrified growth medium bottle and inoculate into a fresh growth medium bottle. This transfer should be performed every ~7 days. |  |
| NOTE: Check that there is **no** excess skin or film formed in the growth media. Discard medium if found. |  |

| **Preparation of bacteria pellets (Duration: 1 day)** | |
|---|---|
| Once $NO_3^-$ in medium is denitrified, it is ready to be concentrated.<br><br>Autoclave centrifuge tub at 120 °C for 30 min. In laminar hood, dispense denitrified growth medium into 4 × 750 mL tubs, filling each with 600 mL. Centrifuge at 4000 rpm for 40 minutes at 20 °C. |  |
| Carefully discard supernatant into a beaker in the laminar hood, not to disturb the pellets of bacteria at the bottom of the tube. |  |
| Rinse the pelleted bacteria by filling each tub with 60 mL of resuspension medium. Centrifuge at 4000 rpm for 20 minutes at 20 °C. Again, carefully discard supernatant into a beaker in the laminar hood. |  |
| Finally, re-suspend the pellets with 60 mL of fresh resuspension medium. Vortex to ensure homogeneous mixing and dispense the concentrated bacteria from all 4 tubs into 500 mL narrow mouthed Schott Duran bottle. Cap with a # 49 Suba-Seal soaked in 70% ethanol overnight. |  |

| | |
|---|---|
| The final volume should be ~240 mL of 10 × concentrated bacteria. Label bottle with "*Pseudomonas aureofaciens*, 10x", date of production and name of technician. |  |

#### *2.3.3.1. Conversion of nitrate to nitrous oxide in samples*

Prior to conversion, dilute or concentrate the water samples to ±350µmol/L $NO_3^-$ so that the $N_2O$ produced is within the linear range of the analyser. Number of samples can be changed by increasing or decreasing purging capacity on the purging block. Processes can be performed under usual laboratory conditions.

The Soil and Water Management & Crop Nutrition Laboratory designed and built a custom purging block that allows for precise regulation of purging gas flow and larger sample processing. The technical specifications and usage manual of the Gas-through-Liquid Purging Block (GLPB) can be found in the Technology Annex in Section 2.5.

### 2.3.4. Equipment

**Materials for purging**

- 16 × 60 mL glass vials, washed and autoclaved;
- 16 × grey butyl septa, autoclaved;
- 16 × aluminium crimp seals;
- Aluminium crimp sealer;
- 16 × short 26G sterile needles (Long enough to pierce grey butyl septa but not higher than liquid when vial is overturned);
- 16 × long 26G sterile needles (Long enough to break the liquid surface when vial is overturned);
- 1 × sterile syringe (Any volume between 5-10 mL);
- 80 mL concentrated *Pseudomonas aureofaciens* bacteria culture;
- Purging block with $N_2$ gas connection

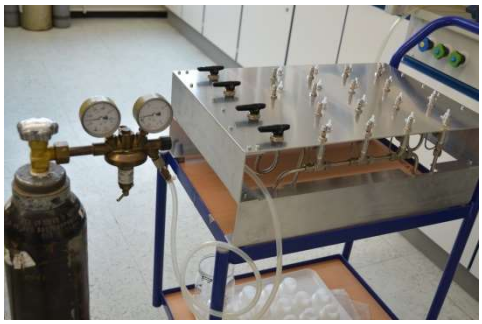
**Materials for reaction with water samples**

- 16 × 26G sterile needles (Length not relevant, as long as water sample can be injected);
- 16 × sterile syringes (Any volume below 5 mL);
- Vial holder sufficient for sample vials

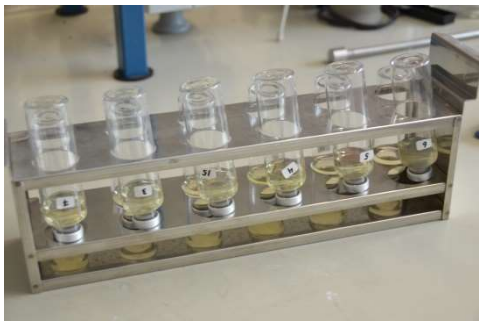
**Materials for sample injection**

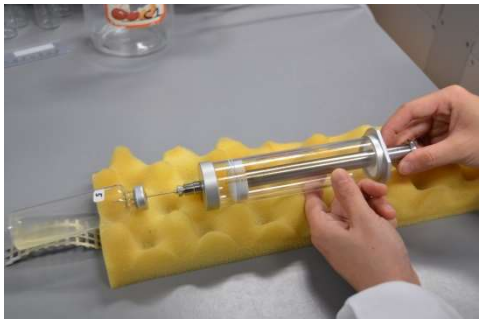
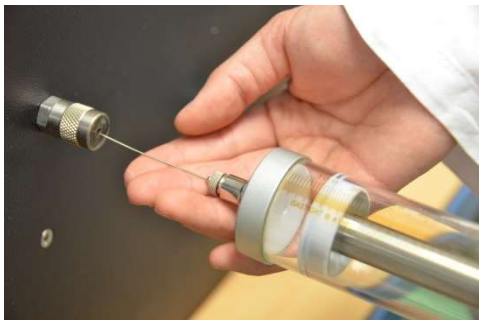
- Incubated water samples;
- Gas tight syringe (at least 10 mL)

### 2.3.5. Procedure

| **Purging concentrated bacteria (3-4 hours, depending on flow of $N_2$ gas)** | |
|---|---|
| Under sterile conditions. assemble autoclaved vial, grey butyl septa and crimp seal with aluminium cover.<br><br>Extract 4 mL of concentrated bacteria with sterile syringe and needle, inject into vials. |  |
| Ensure $N_2$ gas connection to purging block is secured and sufficient for at least 3 hours of purging. |  |
| Attach short* 26G needles onto purging ports, this is the purging needle. Remove protective cover only when placing sample vials on top.<br><br>** Length of needle is dependent on height of bacteria and water sample in vial. Ensure that needle is short enough that it does not break the surface of the aqueous mixture when the vial is overturned.* |  |

| | |
|---|---|
| Attach long* 26G needles into vials. This is the pressure release needle.<br><br>When inserting the pressure release needle, ensure bottle is on the side before pushing the needle all the way through so that the concentrated bacteria will not enter the needle, causing loss of bacteria.<br><br>** Length of needle is dependent on height of sample vial. Ensure that needle is long enough to reach top of the vial.* |  |
| Turn on purging system to a low flow to prevent backflow of bacteria.<br><br>Remove protective cover of bubbling needle and insert purging vial.<br><br>Ensure purging needle is covered by bacteria concentrate, and pressure release needle is not in contact with bacteria. Increase flow of $N_2$ gas and maintain a consistent flow. |  |
| To ensure the vial does not fall over, a makeshift support can be placed. Purge for at least 3 hours. |  |
| Upon completion of purging, remove vials from purging block.<br><br>To create a slight overpressure and ensure no backflow of ambient air, decrease flow of $N_2$ gas, swiftly remove pressure release needle, followed by purging needle. |  |

| **Injection of water samples and overnight incubation** | |
|---|---|
| Label each vial according to sample numbering.<br><br>With sterile syringe and 26G needle, inject approximately 1 mL of ~350µmol/L $NO_3^-$ water sample to each sample vial. |  |
| Store overturned vials on a vial holder and incubate overnight. |  |

| **Injection of $N_2O$ gas samples into $N_2O$ Laser Absorption Spectroscope Analyser** | |
|---|---|
| Slowly extract 10 mL gas sample from vials using gas tight syringe. Caution should be taken to avoid liquid from entering the needle. |  |
| When injecting gas sample, guide the needle through the port with your hands to prevent the needle from bending under force.<br><br>Once measurements are performed, write sample name, results and volume of gas injected for record purposes. |  |

## 2.4. CONDITIONING, NORMALIZATION AND ASSOCIATED CALCULATIONS

The methods below describe the normalization required for data analysis and measurement. As with the identical treatment concept, it is imperative that all standards, samples and reference materials in one measurement run to use the same batch of concentrated *Pseudomonas aureofaciens* bacteria. Using bacteria from different batches within a run may introduce analytical uncertainties in the measurement. All blanks, standards and reference materials are processed exactly the same way as the samples.

At least 3×stable isotope reference materials should be analysed with each batch of samples. Due to relatively high analytical uncertainty of the denitrification method it is recommended to use at least two international nitrate reference materials (e.g. USGS35, USGS32) and one laboratory reference, each replicated two times. The international reference materials should be used for normalization of all raw data to the international scale (Air $\delta^{15}N = 0‰$). The laboratory standard that has been calibrated against the international reference materials and has $\delta^{15}N$ value close to those expected for samples should be used as a check/quality control standard.

### 2.4.1. Equipment

**Materials for standard and blank**

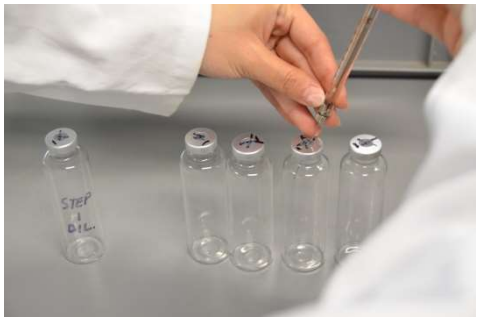
- International stable isotope $NO_3^-$ reference materials at 350µmol/L $NO_3^-$ (e.g. USGS32 and USGS34 or USGS35).
- Laboratory stable isotope $NO_3^-$ reference material at 350µmol/L $NO_3^-$
- 2 mL deionized or ultrapure water (same water which has been used to prepare reference materials) for blanks, the water has to be $N_2O$-free.
- 11 × autoclaved 60 mL crimp sealed glass vials with 4 mL concentrated bacteria injected.
- Laser Absorption Spectroscope Analyser.
- Purging block with stable $N_2$ gas connection.

**Materials for conditioning**

- 1 × gas sampling bag 0.5-1 L (each conditioning run requires approx. 0.5 mL $N_2O$ gas);
- Pure $N_2O$ gas;
- Pure $N_2$ gas connected to purging block;
- 5 × 60 mL gas tight glass vials;
- 1 × gas tight syringe (0.5 – 1 mL);
- Laser Absorption Spectroscope Analyser

### 2.4.2. Procedure

| **Creating standard and blank** | |
|---|---|
| Under sterile conditions, crimp seal 11 autoclaved vials and inject 4 mL concentrated bacteria into all. Purge following the procedures in Section 2.3.5.<br><br>Using sterile syringes, prepare as below:<br><br><u>Standards*:</u><br>• 2 × vials with 1 mL of USGS35 each (or USGS34)<br>• 2 × vials with 1 mL of USGS32 each<br><br>**All standards have concentration of 350µmol/L $NO_3^-$ and are prepared with deionized or ultrapure $N_2O$-free water.*<br><br><u>Blanks:</u><br>• 2 × vials with 1 mL of deionized or ultrapure N2O-free water each<br><br>Label vials accordingly and allow for overnight denitrification. Extract 10 mL gas when ready and measure with the $N_2O$ Laser Absorption Spectroscope Analyser. |  |

| **Conditioning of $N_2O$ Laser Absorption Spectroscope Analyser** | |
|---|---|
| The $N_2O$ Laser Absorption Spectroscope Analyser should be conditioned and calibrated using 50 ppm $N_2O$ gas in synthetic air or if available in $N_2$. Preferably a large tank sufficient for several months should be used. If desired concentration is not available it can be prepared following the procedure as below.<br><br>Note: This gas should also be used between samples to determine drift of the analyser. |  |
| Flush three times and fill a gas sampling bag with pure $N_2O$ gas. Flush the crimp sealed glass vials with pure $N_2$ gas.<br><br>Extract 0.5 mL pure $N_2O$ gas from the gas sampling bag with gas tight syringe and inject into one crimp sealed glass vial. Label vial as "Step dilution 1". |  |
| From "Step dilution 1" vial, extract and inject 0.2 mL gas in each of the remaining four vials. |  |
| Using a gas tight syringe, inject 10 mL diluted $N_2O$ gas into the analyser and measure the $\delta^{15}N$ of $N_2O$. Repeat at least four times, or until certain that the readings have stabilized (as a "dummy sample" for confirming good performance of the analyser). Export data from the analyser as per manufacturer's protocol. |  |

### 2.4.3. Normalization of the raw $\delta^{15}N$ and $\delta^{18}O$ results to the international scale

The raw $\delta^{15}N$ and $\delta^{18}O$ results obtained for $N_2O$ samples measured with the Laser Absorption Spectroscope Analyser require normalization to the international stable isotope scale for nitrogen and oxygen. The international scale for $\delta^{15}N$ is defined with respect to atmospheric nitrogen, with $\delta^{15}N$ assigned as 0‰, while the scale for $\delta^{18}O$ is defined with respect to Vienna Standard Mean Ocean Water (VSMOW) water and is also assigned as 0‰. The stable isotope scales are realized using international reference materials, such as the two primary scale anchors IAEA-N1 0.43‰ and USGS32 180‰ for $\delta^{15}N$ [2.6.] and VSMOW 0‰ and SLAP2 -55.5 for $\delta^{18}O$ [2.7.]. The primary reference materials are used for calibration of secondary reference materials, including in-house laboratory reference materials.

In order to normalize the raw $\delta^{15}N$ values of samples, the calibrated reference materials need to be processed and analysed by following identical treatment and chemical matrix matching principles, and using exactly the same protocol as is used for all other samples. Therefore, for a denitrification method, nitrate reference materials should be used at concentrations similar to those present in the samples. Water solutions of three reference materials, each prepared in duplicate, need to be denitrified and the obtained $N_2O$ analysed.

The $\delta^{15}N$ and $\delta^{18}O$ values of the reference materials used for normalization should roughly encompass the whole range of values expected for the samples and preferably the whole range of the values observed in nature for the isotope composition of the chemical compound of interest [2.8.]. In this particular case, the range of $\delta^{15}N$ values observed in nature for nitrates lies roughly between -25 and +150‰ and between -10 and +80‰ for $\delta^{18}O$ [2.9.]. The third reference material used for checking and quality control should have $\delta^{15}N$ and $\delta^{18}O$ values close to the values of the analysed samples. Typically, USGS32, USGS34 or USGS35 reference materials are used, along with in-house calibrated laboratory reference materials, using two replicates for all materials. The results of analyses of two of these reference materials (e.g. USGS32 and USGS34 for $\delta^{15}N$ and USGS34 and USGS35 for $\delta^{18}O$) should be used for a multi-point normalization by constructing a linear regression model. The third reference material (e.g. USGS35 for $\delta^{15}N$ and USGS32 for $\delta^{18}O$) should be used as a quality control [2.10.]. The linear regression model for normalization should be constructed as shown in the example presented in Fig. 2.1 and Table 2.1.

*Example of the normalization procedure*
This example is provided for normalization of $\delta^{15}N$ values. The same procedure and the same reference materials can be used for normalization of $\delta^{18}O$ values. The reference materials listed below have also been calibrated and reported for $\delta^{18}O$ of nitrates [2.11.], and the current up-to-date values for all reference materials can be confirmed on the IAEA web site: https://nucleus.iaea.org/rpst/referenceproducts/referencematerials/Stable_Isotopes/index.htm.

**Step 1.** Analyse the two replicates of each of the two well-calibrated reference materials (e.g. USGS34 and USGS32). As a quality control reference material, analyse two replicates of one well-calibrated in-house laboratory reference material (or e.g. USGS35). Analyse your samples.

**Step 2.** Check that the difference between the $\delta^{15}N$ of the two replicates for each reference material is within the accepted combined analytical uncertainty of the method (e.g. <0.5‰, 1 st.dev.). Consider the quality of the measurements, technical conditions, and laboratory observations and eventually identify and discard outliers.

**Step 3.** Use the raw results obtained for the two USGS34 and two USGS32 reference materials (four points in total) to construct a linear regression model for the normalization procedure, using the following equation:

$$\delta^{15}N_{true} = a \times \delta^{15}N_{measured} + b \qquad (2.1)$$

where *true* is the known $\delta^{15}N$ value from a reference material information sheet provided by the reference distributor (e.g. IAEA), *measured* is the $\delta^{15}N$ value obtained during analysis (using the Laser Absorption Spectroscope Analyser), $a$ is the slope and $b$ is the intercept.

**Step 4.** Confirm that your slope ($a$) is within the range of approximately 0.9 to 1.1.

**Step 5.** Normalize the measured $\delta^{15}N$ values of your checking reference material (here it would be USGS35) to the international scale, as this would be unknown. Multiply $\delta^{15}N_{measured}$ by the slope ($a$) and add intercept ($b$). Confirm that the obtained normalized $\delta^{15}N$ value does not differ significantly from the true value reported on the reference material information sheet provided by the reference distributor. The acceptable difference is equal to the accepted combined analytical uncertainty for the used method (e.g. ±0.5‰ for the denitrification method).

**Step 6.** If a difference greater than two analytical uncertainties is obtained for the $\delta^{15}N$ values between replicates, or between the values calculated during normalization and the true values expected for the checking reference material, then the entire set of standards and samples should be rejected. If the results and the uncertainties are in the acceptable range, then normalize all results for your samples following the same procedure described in Step 5.

*Note that the slope and the intercept of normalization equations and values will vary depending on the system, technique and reference materials used.*

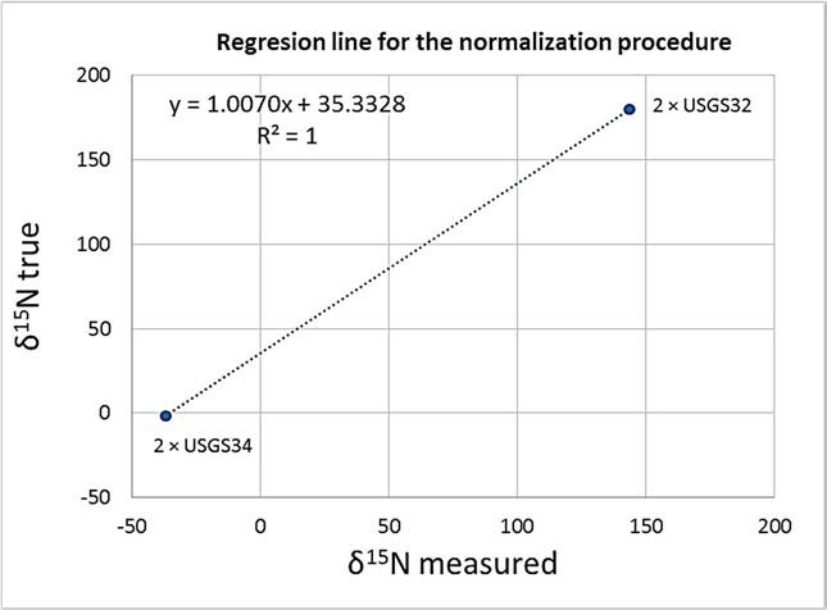



*FIG. 2.1. Example of the linear regression used for the normalization procedure (four points in total have been used, not visible due to the scale of the plot), for details see Steps 1 to 6 and Table 2.1.*

TABLE 2.1. EXAMPLE OF THE NORMALIZATION PROCEDURE, FOR DETAILS SEE STEPS 1 TO 6.

| **Normalization** | **$\delta^{15}N$ measured** | **$\delta^{15}N$ true** | **$\delta^{15}N$ difference between repl.** | | |
|---|---|---|---|---|---|
| USGS 32 rep1 | 143.91 | 180 | 0.49 | | |
| USGS 32 rep2 | 143.42 | 180 | | | |
| USGS 34 rep 1 | -37.14 | -1.8 | -0.53 | | |
| USGS 34 rep 2 | -36.61 | -1.8 | | | |
| | slope | 1.0070 | | | |
| | intercept | 35.3328 | | | |
| | $R^2$ | 1.0000 | | | |
| **Check** | **$\delta^{15}N$ measured** | **$\delta^{15}N$ true** | **$\delta^{15}N$ difference between repl.** | **$\delta^{15}N$ normalized** | **$\delta^{15}N$ difference normal-true** |
| USGS 35 rep1 | -32.51 | 2.7 | -0.65 | 2.60 | -0.10 |
| USGS 35 rep2 | -31.86 | 2.7 | | 3.25 | 0.55 |
| | | | | | |
| **Samples** | **$\delta^{15}N$ measured** | | | **$\delta^{15}N$ normalized** | |
| Sample 1 | -35.4 | | | **-0.31** | |
| Sample 2 | -31.2 | | | **3.92** | |
| Sample 3 | -25.4 | | | **9.76** | |

## 2.5. TECHNOLOGY ANNEX

### 2.5.1. Scope and field of application

The Gas-through-Liquid Purging Block (GLPB), designed and constructed at the Soil and Water Management & Crop Nutrition Laboratory, allows precise regulation of the purging gas flow at each of its ports (16 ports in total). It also allows processing larger sample batches

conveniently. The GLPB has been designed to drive gas through bottled liquids. One of the main applications is driving out microorganisms related respiration products in an effective and consistent manner.

*FIG. 2.2. The GLPB designed and created at the SWMCNL, in collaboration with the Office and Safeguards Analytical Services' Technical Support Team. The block has a capacity of 16 ports, and is useful for purging gases from samples.*

*FIG. 2.3. GLPB in action. Note the gas bubbles in the bottle on the right. Makeshift supports for the other bottles while purging.*

### 2.5.2. Advantages

Advantages of the GLPB are as below:

- Time efficient and flexible in processing samples: one to 16 samples can be processed simultaneously;
- Secure handling of pressured gas;
- Flexible design, which fits bottle sizes from 25 ml to 500 ml;

- Possibility of purging different volumes at the same time;
- Gas throughput adjustable from millilitres to several litres per minute;
- Suitable for all non-corrosive and non-explosive gases;
- Precise regulation of gas flow for each port can create uniformity and saves purging gas;
- Uniformity in sample handling is possible;
- Compact dimensions: length × width × height = 60 cm × 40 cm × 35 cm;
- Low weight due to its aluminium frame

Due to the design of the GLPB system, several GLPBs can be connected in series, which allow purging of a multiple of 16 samples at one time. Thus, even larger numbers of samples can be processed conveniently.

### 2.5.3. Equipment description

*FIG. 2.4. GLPB layout. Four main lines (Yellow), each equipped with a 90°ball valve*

The GLPB is a gas distribution system designed to deliver a constant, precisely adjustable gas flow to each of its 16 ports. Gas enters through a main line, and is diverted to four side lines; each of those can be opened/closed by a 90° ball valve. Every side line has four ports. Ports are equipped with precision needle valves which allow finest regulation of gas flows. Due to the construction, each port can be addressed with gas individually, so any number of bottles between one and 16 can be purged in one go.

Gas outlets can be equipped with injection needles. Suitable length and diameter of the needles depend on bottles used and their lids (septa). Bottles are placed upside down on the gas ports. Beside the gas-delivering needle, a second needle is inserted through the septum, acting as gas escape route from the bottle. This needle needs to be as long as the bottle height, the tip shall end well above the liquid's surface.

Handling injection needles requires great care! It is recommended to insert the needles into septum and bottle, before placing the assembly (bottle with liquid to purge, plus lid, septum and needles) on the GLPB port.

*FIG. 2.5. GLPB on secure support in the laboratory.*

Ports should drive gas before the assembly is put on in order to avoid liquid entering into the port through the port needle. When finished, the assembly should be disconnected prior to turning off the port valve.

The gas supply should deliver gas at a constant rate, with a pressure of maximal two bars.

### 2.5.4. Modus of use

1. Put GLPB on a safe support;
2. Connect to gas supply;
3. Do all assemblies (bottle with liquid to purge, plus lid, septum and needles);
4. Open the valve of the port you want to put the assembly on
5. Put the assembly on the port; adjust the gas flow to the desired rate. Do that for all assemblies;
6. When purging time is over, close each port valve after pulling off an assembly;
7. Once all assemblies are disconnected, turn off-gas supply.

### 2.5.5. GLPB construction materials and part list

Parts specified in here with article numbers and provider names reflect the items used for constructing the prototype. Any other items meeting the technical specifications of the items used for this particular construction can be utilized to build a GLPB system.

Costs listed as they were in 2016, in Austria and may vary in time and geographical location.

Drilling holes into the aluminium board for inserting the port valves as well as producing the redesigned needle adapters, which are now made out of aluminium, was done by the Technical Support Team from the Office of Safeguards Analytical Services.

Injection needles, septas, lids and bottles are excluded from the Table 2.2. as these items may vary due to the experimental set-up.

*FIG. 2.6. Finished tubing. Main lines and side lines of the gas supply.*

*FIG. 2.7. Precision valve knobs and needle connection adapters of the GLPB*

TABLE 2.2. GLPB CONSTRUCTION MATERIALS AND PART LIST

| Article number (Swagelok) | Item Description | Amount | Price per unit (EUR) | Sum (EUR) |
|---|---|---|---|---|
| SS-200-61 | Stainless Steel Swagelok Tube Fitting, Bulkhead Union, 1/8 in. Tube OD | 16 | 27 | 432 |
| SS-8M0-7-2 | Stainless Steel Swagelok Tube Fitting, Female Connector, 8 mm Tube OD×1/8 in. Female NPT | 3 | 14 | 42 |
| SS-8M0-6 | Stainless Steel Swagelok Tube Fitting, Union, 8 mm Tube OD | 1 | 15 | 15 |
| SS-8M0-3 | Stainless Steel Swagelok Tube Fitting, Union Tee, 8 mm Tube OD | 3 | 31 | 93 |
| SS-43GS8MM-1466 | Stainless Steel 1-Piece 40G Series Ball Valve, 1.5 Cv, 8 mm Swagelok Tube Fitting, No Lube, SC-11 Cleaned | 4 | 174 | 696 |
| SS-8M0-3-4TTF | Stainless Steel Swagelok Tube Fitting, Female Branch Tee, 8 mm Tube OD×8 mm Tube OD×1/4 in. Female NPT | 12 | 64 | 768 |
| SS-8M0-7-2 | Stainless Steel Swagelok Tube Fitting, Female Connector, 8 mm Tube OD×1/8 in. Female NPT | 1 | 13 | 13 |
| SS-SM2-S2-A | Stainless Steel Low Flow Angle Pattern Metering Valve, 1/8 in. MNPT×1/8 in. Swagelok Tube Fitting | 16 | 141 | 2256 |
| SS-200-1-2 | Stainless Steel Swagelok Tube Fitting, Male Connector, 1/8 in. Tube OD×1/8 in. Male NPT | 16 | 9 | 144 |
| SS-202-61 | 316 Stainless Steel Jam Nut for 1/8 in. Bulkhead Swagelok Tube Fitting | 16 | 4 | 64 |
| SS-T2-S-028-6ME | 316/316L Stainless Steel Seamless Tubing, 1/8 in. OD×0.028 in. Wall | 6 | 10 | 60 |
| SS-T8M-S-1.0M-6ME-S | 316/316L Stainless Steel Seamless Tubing, 8 mm OD×1.0 mm Wall | 6 | 10 | 60 |
| <none> | Aluminium U-Profile, 40x100x40x3 mm L=1500 mm | 1.5 | 15 | 22.5 |
| <none> | Washers, Stainless Steel, M4 | 50 | 0.1 | 5 |
| <none> | Aluminium Board, rolled, 3 mm thick, 60 times 40 centimeters, Protective Foil on one side | 2 | 23 | 46 |
| <none> | Hexagon socket Screws, Stainless Steel, M4 × 20mm | 50 | 0.25 | 12.5 |
| <none> | Nuts, self-locking, Stainless Steel, M4 | 50 | 0.20 | 10 |
| <none> | Needle adapters | 16 | n.a | n.a |
| | | | Sum | 4739 |

## REFERENCES TO CHAPTER 2


[2.1] KENDALL, C., Tracing nitrogen sources and cycling in catchments. Isotope Tracers in Catchment Hydrology. (1998) 519-576.

[2.2] PANNO, S.V., HACKLEY, K.C., HWANG, H.H., KELLY, W.R., Determination of the sources of nitrate contamination in karst springs using isotopic and chemical indicators. Chemical Geology **179** (1) (2001) 113-128.

[2.3] FOGG, G.E., ROLSTON, D.E., DECKER, D.L., LOUIE, D.T., GRISMER, M.E., Spatial variation in nitrogen isotope values beneath nitrate contamination sources. Ground Water **36** (3) (1998). 418-426.

[2.4] CASCIOTTI, K.L., SIGMAN, D.M., HASTINGS, M.G., BÖHLKE, J.K., HILKERT, A., Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. Analytical Chemistry **74** (19) (2002) 4905-4912.

[2.5] SIGMAN D.M., CASCIOTTI K.L., ANDREANI M., BARFORD C., GALANTER M., BAHLKE J.K., A Bacterial Method for the Nitrogen Isotoic Analysis of Nitrate in Seawater and Freshwater. Analytical Chemistry **73** (2001) 4145-4153.

[2.6] BÖHLKE J.K., COPLEN, T.B., Interlaboratory comparison of reference materials for nitrogen-isotope-ratio measurements, Reference and intercomparison materials for stable isotopes of light elements, IAEA TECDOC 825: International Atomic Energy Agency, Vienna (1995) 51-66.

[2.7.] COPLEN T.B., New guidelines for reporting stable hydrogen, carbon, and oxygen isotope-ratio data. Geochimica et Cosmochimica Acta **60** (1996) 3359.

[2.8.] SKRZYPEK G., SADLER R., A strategy for selection of reference materials in stable oxygen isotope analyses of solid materials. Rapid Commun. Mass Spectrom **25** (2011) 1625-1630.

[2.9.] COPLEN T. B., HOPPLE J. A., BÖHLKE J. K., PEISER, H. S., RIEDER S. E., KROUSE H. R., ROSMAN K. J. R., DING T., VOCKE Jr R. D., RÉVÉSZ K. M., LAMBERTY A., TAYLOR P., BIÈVRE D., Compilation of Minimum and Maximum Isotope Ratios of Selected Elements in Naturally Occurring Terrestrial Materials and Reagents. US Geological Survey Water Resources Investigation. (2002) Vol. Report 01-4222.

[2.10.] SKRZYPEK G., Normalization procedures and reference material selection in stable HCNOS isotope analyses - an overview. Analytical and Bioanalytical Chemistry **405** (2013) 2815-2823.

[2.11.] BRAND W.A., COPLEN T. B., AERTS-BIJMA A. T., BÖHLKE J. K., GEHRE M., GEILMANN H., GRÖNING M., JANSEN H. G., MEIJER H. A. J., MROCZKOWSKI S. J., QI H., SOERGEL K., STUART-WILLIAMS H., WEISE S. M., WERNER R. A., Comprehensive inter-laboratory calibration of reference materials for δ18O versus VSMOW using various on-line high-temperature conversion techniques. IAEA-TECDOC-825. International Atomic Energy Agency, Vienna, (1993) 51–66.


## BIBLIOGRAPHY TO CHAPTER 2


McILVIN, M.R., CASCIOTTI, K.L., Technical updates to the bacterial method for nitrate isotopic analyses. Analytical Chemistry **83** (5) (2011) 1850-1856.

# CHAPTER 3. MICRO-DIFFUSION OF $^{15}N$ ENRICHED AMMONIUM AND NITRATE SAMPLES

M. HEILING

## 3.1. SCOPE AND FIELD OF APPLICATION

The micro-diffusion method is used to determine the stable nitrogen isotope composition ($^{15}N/^{14}N$) of ammonium and nitrate/nitrite in liquid samples. In this technique, ammonium and nitrate/nitrite are isolated prior to analysis of $^{15}N/^{14}N$ with the isotope ratio mass spectrometry (IRMS) or emission spectroscopy (ES). Theoretically all types of liquids can be analysed but depending on the considered N-pool, pre-extractions can be necessary. For soil inorganic N studies, KCl soil extracts need to be prepared. The method cannot discriminate between nitrate and nitrite, therefore the obtained results will reflect the sum of both components respectively to their relative concentrations, but usually nitrite concentrations are very low or negligible. To simplify the reading, the term nitrate will be used instead of nitrate/nitrite in this text. This SOP will focus on sample preparation for IRMS, for ES details can be found in [3.3].

Analyses of $^{15}N$ enriched nitrate and ammonium can be applied in many studies of inorganic N-pool, e.g.:

- N-leaching studies in micro-plots;
- N-balance studies;
- Mineralization-immobilization turnover in soil N-pools;
- Fertilizer use efficiency studies

## 3.2. PRINCIPLE

The method can be used to determine $^{15}N/^{14}N$ of ammonium ($NH_4^+$) and nitrate ($NO_3^-$) separately. In this case the pH of the sample is increased to pH>9, what leads to diffusion of ammonia ($NH_3$) (Equation 3.1). The liberated $NH_3$ is collected on an acidified glass fibre paper which is sealed between two gas permeable polytetrafluoroethylene (PTFE) strips and reacts with the acid to the corresponding ammonium salt which later on will be used for IRMS analysis. In the second step, Devarda's alloy is used as a reducing agent and catalyst to convert the remaining nitrate in the alkaline solution to $NH_3$ (Equation 3.2), which again is trapped on a separate acidified disc and used for IRMS analysis.

Alternatively, the total inorganic N-pool (ammonium and nitrate) can be determined. In that case, pH increase and nitrate conversion with Devarda's alloy are done in one step.

$$NH_4^+ + OH^- \rightarrow NH_3 + H_2O \quad (3.1)$$

$$3\ NO_3^- + 8\ Al + 5\ OH^- + 18\ H_2O \rightarrow 3\ NH_3 + 8\ [Al(OH)_4]^- \quad (3.2)$$

## 3.3. REAGENTS

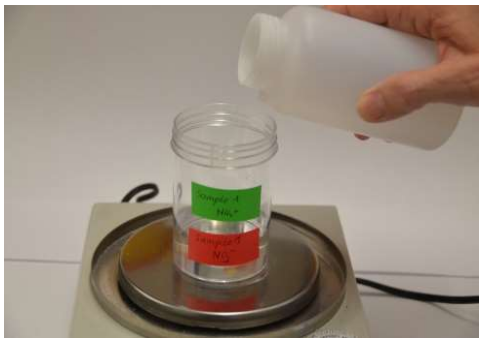
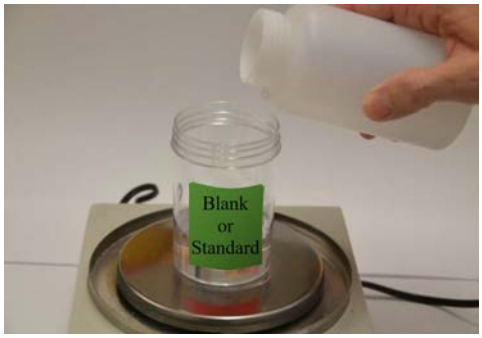
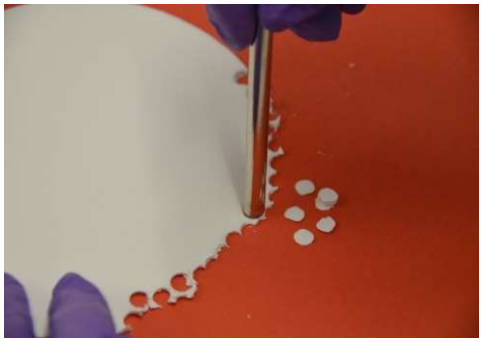
- 2.5 M potassium bisulphate (68g $KHSO_4$ dissolved in 200 ml distilled water);
- Devarda's alloy;
- Magnesium oxide (MgO),
- $NO_3^-$ and $NH_4^+$ in-house laboratory reference materials, which have been calibrated against primary reference materials such as IAEA 305 or 311, with similar enrichments as samples but not higher than ~5 atm%
- Ethanol to clean tweezers

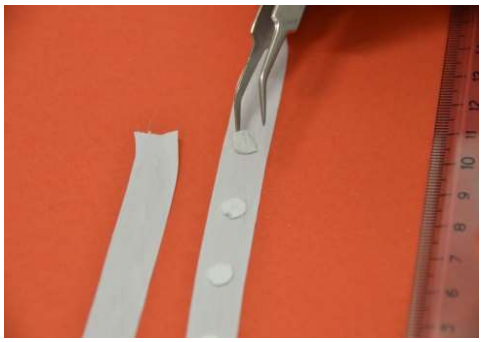
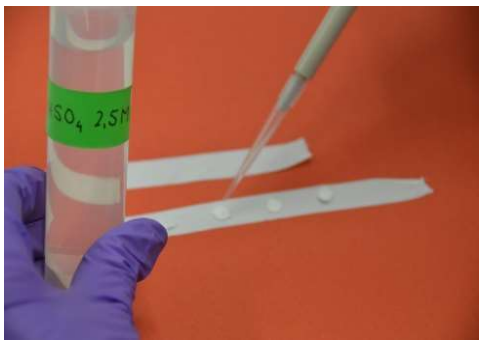
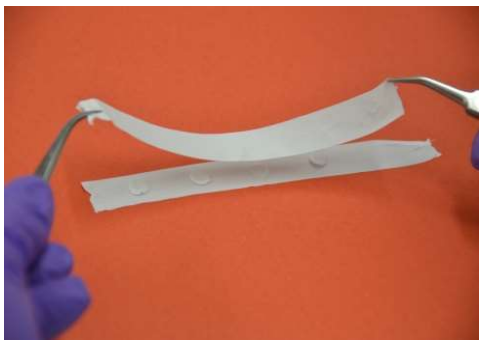
## 3.4. EQUIPMENT

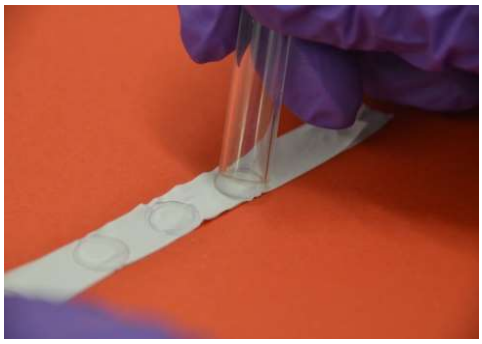
- Balance with 0.01 g readability.
- Gas tight, disposable plastic container, e.g. centrifuge vials, urine beakers or liquid scintillation vials.
- Quartz microfiber filter discs (e.g. Whatman, CAT-Nr. 1851-150), cut into circles using a paper punch or similar.
- PTFE strips, e.g. industrial teflon tape (1/2''×12 MT×0.075).
- Micro pipette, 10µL and tips.
- Brush.
- Sharp scissors.
- Tin capsules, 5x8 mm.
- Tweezers.
- Micro-plate rack with lid.
- Desiccator with silica gel and conc. sulfuric acid.
- Laboratory gloves to avoid sample contamination.
- Level scoops for MgO and Devarda's alloy if available.

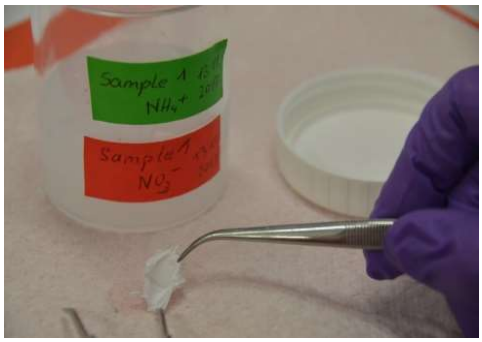
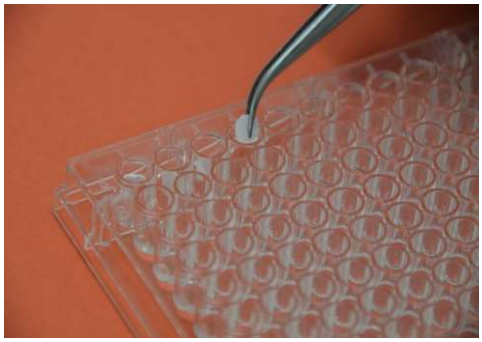
## 3.5. PROCEDURE

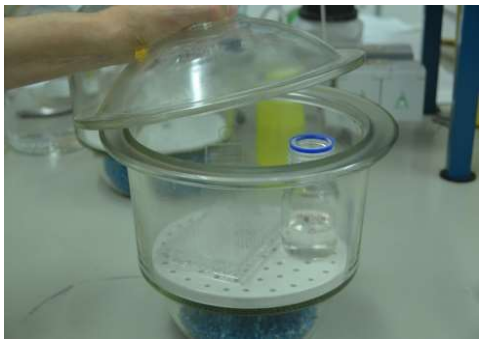
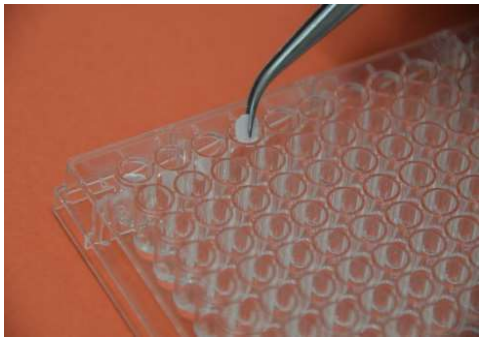
| **Procedure for ammonium and nitrate $^{15}N$-analyses extracted separately** | |
|---|---|
| **Step 1: Labeling of jars**<br><br>Label gas tight jars with labels for each:<br><br>Sample name – $NH_4^+$, date<br><br>and<br><br>Sample name – $NO_3^-$, date |  |

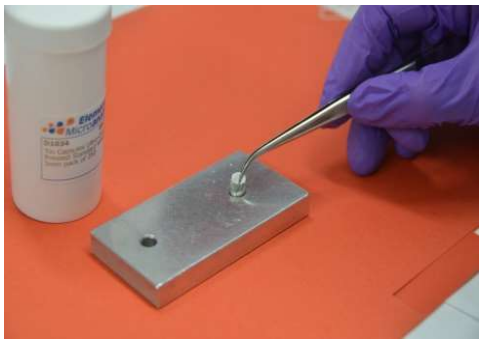
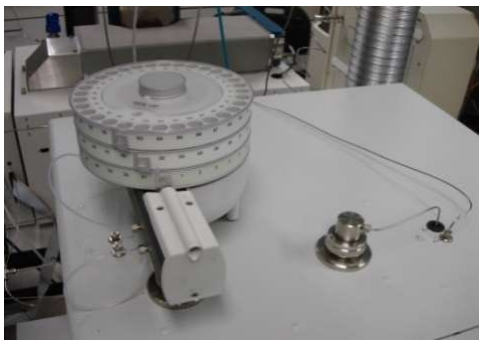
| | |
|---|---|
| **Step 2: Weighing/pipetting sample aliquot**<br><br>Weigh or pipette an aliquot of sample, containing the target amount of N, required by the IRMS (0.05 - 0.20 mg of N).<br><br>For typical KCl soil extracts this is usually 50 mL extract, however, in low N soils this may be up to 100 mL. |  |
| **Step 3: Blanks and standards**<br><br>Process blanks in the same way as the samples. Analyse standards for ammonium and nitrate at the same concentration range as the samples and with similar $^{15}N$ enrichments. Follow the procedure the same way as for the samples.<br>Note: If KCl extracts are processed, use the same KCl solution for the blanks, standards and samples. |  |
| **Step 4: Preparation of acidified trap**<br><br>Punch out discs of the quartz glass fibre filter paper with a diameter smaller than the PTFE tape and store them in a jar. Gloves are recommended to avoid contamination. |  |
| Tip: The easiest way to collect the small discs, is to pick them with a needle. |  |

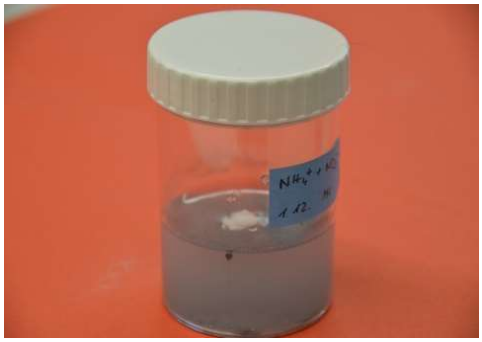
| | |
|---|---|
| Flatten PTFE tape with a brush and cut to a length of approximate 10-15 cm. |  |
| Place quartz glass discs on the tape using tweezers. The distance between the discs should be approximate 2 cm. |  |
| Pipette 10µL of 2.5M $KHSO_4$ onto each quartz glass fibre disc. |  |
| Cover the filter disc with a second layer of PTFE tape. |  |

| | |
|---|---|
| Seal both PTFE layers by pressing with a round shaped test tube with smooth edges. Make soft swirling movements in a circular motion to compress the PTFE tapes and create a perfect seal. |  |
| Cut-off single acid trap envelopes and use them immediately after preparation. |  |
| **Step 5: Trapping ammonium**<br>Add one acid trap per sample/blank/standard. Then add 0.1g MgO and close the vial immediately.<br>Note: You can prepare a level scoop with a volume equivalent to 0.1g MgO. |  |
| Acidic solutions could require more MgO, check pH of a spare sample, pH needs to increase to pH>9. |  |

| | |
|---|---|
| Leave samples at room temperature for 5 to 7 days. |  |
| **Step 6: Removal of acid traps**<br><br>After 5 to 7 days all ammonium should be absorbed as ammonium salt on the acid trap.<br>Use tweezers to take out the PTFE envelopes and place them on a clean paper tissue. Open the traps and remove the upper PTFE layer with a second pair of tweezers. |  |
| Clean tweezers with ethanol after each sample to avoid cross contamination. |  |
| Place the quartz filter papers in a micro-plate rack. Record the position of the samples.<br><br>Note: you can tape the sample label stickers on a sheet with position numbers (e.g. A1, A2 …) |  |

| | |
|---|---|
| Put the samples for drying in a desiccator with silica gel and a bottle containing concentrated sulphuric acid. |  |
| **Step 7: Trapping nitrate**<br><br>Add another acid trap per sample. Then add 0.1g Devarda's alloy and close the vial immediately.<br>Note: You can prepare a level scoop with a volume equivalent to 0.1g Devarda's alloy.<br><br>Devarda's alloy must be treated as toxic waste, containing heavy metals. |  |
| Leave samples at room temperature for 5 to 7 days. Remove acid traps as described in step 6. |  |
| Place the quartz filter papers on the PTFE tapes or in micro-plate racks. Record the position of the samples in the micro-plate rack. Dry them in a desiccator over silica gel and a small bottle containing concentrated sulphuric acid. |  |

| | |
|---|---|
| Transfer the dry filter papers, into tin cups.<br><br>Note: There are two filter papers for each sample/blank/standard – one for the $NH_4^+$ pool and one for the $NO_3^-$ pool. |  |
| Fold them to a ball using tweezers. Do not puncture the tin capsule with the tweezers! |  |
| The samples are now ready for IRMS measurement.<br><br>Note: Check the recovery of the standards –it should be 100% to ensure no isotope fractionation. The N content of the blanks should be < 1µg N or 2% of the sample. |  |

| **Procedure for ammonium and nitrate pool together (total inorganic nitrogen)** | |
|---|---|
| Label gas tight jars with labels for both pools:<br>Sample name $NH_4^+$/ $NO_3^-$, date<br>Follow step 2 to 4 as described above.<br>After putting acid trap, add MgO (100mg) **and** Devarda's Alloy (100mg) in one step.<br>Total inorganic nitrogen will be collected on one trap.<br><br>Continue as mentioned above. |  |

## REFERENCES TO CHAPTER 3


[3.1] BROOKS, P.D., McINTEER, J.M., PRESTON, T., Diffusion method to prepare soil extracts for automated nitrogen-15 analysis. Soil Sci. Soc. Am. J **53** (1989) 1707-1711.

[3.2] SORENSON, P., STEHEN-JENSEN, E., Sequential diffusion of ammonium and nitrate from soil extracts to a polytetrafluoroethylene trap for 15N determination. Anal. Chim. Acta **252** (1991) 201-203.

[3.3] HEILING, M., ARRILLAGA, J., HOOD-NOWOTNY, R., VIDELA, X., Preparation of Ammonium-15N and Nitrate-15N Samples by Microdiffusion for Isotope Ratio Analysis by Optical Emission Spectrometry. Communications in Soil Science and Plant Analysis **37** (2006) 337–346.

## CONTRIBUTORS TO DRAFTING AND REVIEW

| | |
|---|---|
| Heiling, M. | International Atomic Energy Agency |
| Lee Zhi Yi, A. | International Atomic Energy Agency |
| Resch, C. | International Atomic Energy Agency |
| Skrzypek, G. | University of Western Australia |
| Toloza, A. | International Atomic Energy Agency |
| Weltin, G. | International Atomic Energy Agency |
| Yan, T.Z. | International Atomic Energy Agency |

IAEA
International Atomic Energy Agency

No. 25

# ORDERING LOCALLY

In the following countries, IAEA priced publications may be purchased from the sources listed below or from major local booksellers.

Orders for unpriced publications should be made directly to the IAEA. The contact details are given at the end of this list.

## CANADA

***Renouf Publishing Co. Ltd***

22-1010 Polytek Street, Ottawa, ON K1J 9J1, CANADA
Telephone: +1 613 745 2665 • Fax: +1 643 745 7660
Email: order@renoufbooks.com • Web site: www.renoufbooks.com

***Bernan / Rowman & Littlefield***

15200 NBN Way, Blue Ridge Summit, PA 17214, USA
Tel: +1 800 462 6420 • Fax: +1 800 338 4550
Email: orders@rowman.com Web site: www.rowman.com/bernan

## CZECH REPUBLIC

***Suweco CZ, s.r.o.***

Sestupná 153/11, 162 00 Prague 6, CZECH REPUBLIC
Telephone: +420 242 459 205 • Fax: +420 284 821 646
Email: nakup@suweco.cz • Web site: www.suweco.cz

## FRANCE

***Form-Edit***

5 rue Janssen, PO Box 25, 75921 Paris CEDEX, FRANCE
Telephone: +33 1 42 01 49 49 • Fax: +33 1 42 01 90 90
Email: formedit@formedit.fr • Web site: www.form-edit.com

## GERMANY

***Goethe Buchhandlung Teubig GmbH***

Schweitzer Fachinformationen
Willstätterstrasse 15, 40549 Düsseldorf, GERMANY
Telephone: +49 (0) 211 49 874 015 • Fax: +49 (0) 211 49 874 28
Email: kundenbetreuung.goethe@schweitzer-online.de • Web site: www.goethebuch.de

## INDIA

***Allied Publishers***

1st Floor, Dubash House, 15, J.N. Heredi Marg, Ballard Estate, Mumbai 400001, INDIA
Telephone: +91 22 4212 6930/31/69 • Fax: +91 22 2261 7928
Email: alliedpl@vsnl.com • Web site: www.alliedpublishers.com

***Bookwell***

3/79 Nirankari, Delhi 110009, INDIA
Telephone: +91 11 2760 1283/4536
Email: bkwell@nde.vsnl.net.in • Web site: www.bookwellindia.com

## ITALY

***Libreria Scientifica "AEIOU"***

Via Vincenzo Maria Coronelli 6, 20146 Milan, ITALY
Telephone: +39 02 48 95 45 52 • Fax: +39 02 48 95 45 48
Email: info@libreriaaeiou.eu • Web site: www.libreriaaeiou.eu

## JAPAN

***Maruzen-Yushodo Co., Ltd***

10-10 Yotsuyasakamachi, Shinjuku-ku, Tokyo 160-0002, JAPAN
Telephone: +81 3 4335 9312 • Fax: +81 3 4335 9364
Email: bookimport@maruzen.co.jp • Web site: www.maruzen.co.jp

## RUSSIAN FEDERATION

***Scientific and Engineering Centre for Nuclear and Radiation Safety***

107140, Moscow, Malaya Krasnoselskaya st. 2/8, bld. 5, RUSSIAN FEDERATION
Telephone: +7 499 264 00 03 • Fax: +7 499 264 28 59
Email: secnrs@secnrs.ru • Web site: www.secnrs.ru

## UNITED STATES OF AMERICA

***Bernan / Rowman & Littlefield***

15200 NBN Way, Blue Ridge Summit, PA 17214, USA
Tel: +1 800 462 6420 • Fax: +1 800 338 4550
Email: orders@rowman.com • Web site: www.rowman.com/bernan

***Renouf Publishing Co. Ltd***

812 Proctor Avenue, Ogdensburg, NY 13669-2205, USA
Telephone: +1 888 551 7470 • Fax: +1 888 551 7471
Email: orders@renoufbooks.com • Web site: www.renoufbooks.com

**Orders for both priced and unpriced publications may be addressed directly to:**

Marketing and Sales Unit
International Atomic Energy Agency
Vienna International Centre, PO Box 100, 1400 Vienna, Austria
Telephone: +43 1 2600 22529 or 22530 • Fax: +43 1 2600 29302 or +43 1 26007 22529
Email: sales.publications@iaea.org • Web site: www.iaea.org/books

18-03851

International Atomic Energy Agency
Vienna
ISBN 978–92–0–105018–2
ISSN 1011–4289