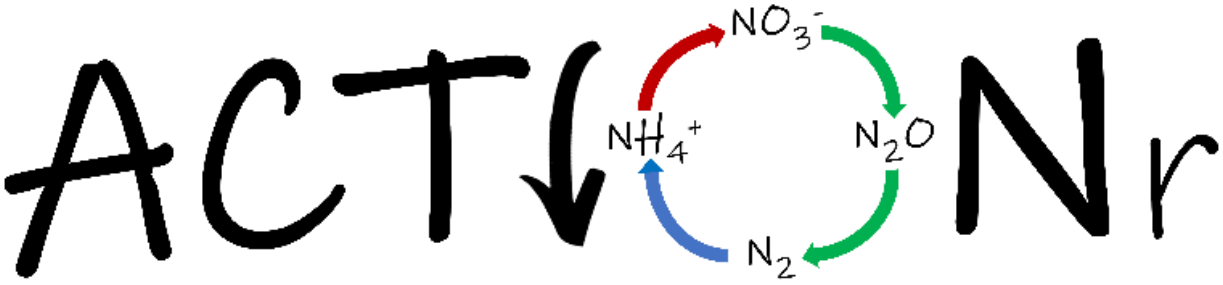

ACTIONr: Research Action Network for Reducing Reactive Nitrogen Losses from Agricultural Ecosystems

Grant Agreement No. 101079299

Deliverable No.	Deliverable Name
D4.1	Modular-based protocol for Ecogenomics application in N cycle

Work Package	WP4: Networking and Sustainability
Lead Partner of Deliverable	University of Thessaly (UTH)
Due date of Deliverable	31-10-2024
Actual Submission date of Deliverable	23-11-2024
Dissemination Level of Deliverable	PU-PUBLIC
Version	1.0

This project has received funding from the European Union's Horizon 2021-2027 research and innovation programme under grant agreement No 101079299.

Participants

No	Name	Logo
1	University of Thessaly (UTH) (Coordinator)	
2	École Centrale de Lyon (ECL)	
2.1	Centre National De La Recherche Scientifique (CNRS)	
3	University of Vienna (UNIVIE)	

Contents

1. Introduction and Purpose 5
2. Modules 5
2.1. Cultivation Protocols 5
2.1.1 Cultivation Protocols for Ammonia Oxidizing Bacteria (AOB) 6
2.1.1.1 Skinner and Walker (SW) Medium 6
2.1.1.2. HK Medium 8
2.1.1.3. 1583 Medium 9
2.1.2 Cultivation Protocols for Ammonia Oxidizing Archaea (AOA) 11
2.1.2.1 Cultivation protocol for Nitrosotalea sinensis Nd2 12
2.1.2.2. Cultivation protocol for Ca. Nitrosocosmicus franklandianus C13 14
2.1.2.2. Cultivation protocol for Nitrososphaera viennensis EN76 17
2.1.3. Nitrite Assays Using a 96-Well Plate Format for Liquid Cultures 19
2.2. Omics Protocols 21
2.2.1. RNA Extraction 21
2.2.1.1. Cell Harvesting for Transcriptomics 22
2.2.1.2. RNA extraction with mirVana™ miRNA Isolation Kit for AOA 22
2.2.1.3. RNA Sample Cleanup and DNA Digestion 23
2.3. Soil Microbial Activity and Abundance Assessment 24
2.3.1. DNA Stable-Isotope Probing (DNA-SIP) 24
2.3.2. Extraction and Quantification of Ammonium, Nitrite, and Nitrate from Soil Samples 27
2.3.3.1. Quantitative PCR (qPCR) for AOB amoA gene 29
2.3.3.2. Quantitative PCR (qPCR) for AOA amoA gene 30
2.3.3.2.1 Alternative Quantitative PCR (qPCR) for AOA amoA gene 31
2.3.3.3. Quantitative PCR (qPCR) for Comammox amoA gene 33
2.3.3.4. Quantitative PCR (qPCR) for Comammox amoA gene in clades A and B 34
2.4. Soil Microbial Diversity Assessment 39
2.4.1. Illumina streamlined Protocol for Library Preparation of AOB, AOA, and Comammox 39
2.4.2. Platform independent protocol for preparing hyper-multiplexed libraries 46
2.4.2.1. Protocol for Ammonia-Oxidizing Bacteria (AOB) 47
2.4.2.2. Protocol for Ammonia-Oxidizing Archaea (AOA) 49
2.4.2.3. Protocol for Comammox amoA gene Amplification 50

2.4.2.4.PCR Clean up and Equimolar Pooling 56
3. Concluding Remarks 56
4. References 57

1. Introduction and Purpose

ACTIONr explores the interactions between nitrification inhibitors (NIs) and nitrogen-cycling microbes across various experimental scales, from *in vitro* assays with single organisms to natural soil microbial communities. Key objectives include decoding the functional pathways of the NIs in ammonia-oxidizing microorganisms (AOM) using dual omics approaches such as transcriptomics and proteomics, developing synthetic microbial communities for activity screening, and assessing the impact of NIs on soil ammonia-oxidizers and greenhouse gas emissions. Additionally, ACTIONr examines the effects of NIs on soil microbiota, considering potential off target ecotoxic effects. Ultimately, the project aims to gather data that support the development of novel, safer, and more effective NIs.

In this context, this modular lab manual consolidates protocols focused on the nitrogen cycle, including applications like Stable Isotope Probing (SIP), ecogenomics, and transcriptomics. By serving as a comprehensive resource, the manual aims to assist researchers in both the ACTIONr project and the wider scientific community by standardizing and optimizing methodologies for studying microbial interactions within the nitrogen cycle. Each module contains established, optimized protocols to ensure adaptability and reproducibility, reflecting the latest advancements as the ACTIONr project evolves.

This manual is designed as a living document, continuously updated to incorporate new data, methodological improvements, and theoretical advances. It provides the foundational protocols utilized by ACTIONr partners, covering cultivation techniques, activity assessments, and microbial diversity analyses. Bioinformatic protocols are intentionally excluded from this manual to maintain a focus on wet lab methodologies. Instead, all scripts and supplementary bioinformatic materials will be directly accessible through the dedicated educational and scientific platform of the project (https://www.actionr.eu/educational-scientific-platform/). Future updates will continue to enhance these protocols to keep pace with ongoing research developments.

2. Modules

2.1. Cultivation Protocols

This module provides cultivation protocols for specific ammonia-oxidizing bacteria (AOB) and archaea (AOA) strains, essential for studying the cellular-level mode of action and activity of tested NIs.

Our approach focuses on cultivating selected AOM strains, including soil-derived betaproteobacteria (*Nitrosospira multiformis* ATCC25196 and *Nitrosomonas communis* Nm2) and Thaumarchaeota archaea (*Nitrososphaera viennensis* EN76, *Nitrosotalea sinensis* Nd2, and *Candidatus* Nitrosocosmicus franklandianus C13). By optimizing culture conditions for each strain in nutrient-specific media, we can assess the biochemical responses to both synthetic and biological NIs, capturing data for subsequent proteomic and transcriptomic analyses.

This module outlines the complete cultivation protocols to ensure consistency in experiments investigating NIs efficacy and the molecular pathways impacted by these inhibitors across diverse AOM groups.

2.1.1 Cultivation Protocols for Ammonia Oxidizing Bacteria (AOB)

Ammonia oxidizing bacteria (AOB) is one of the microbial groups, that participate on the first step of nitrification process, the oxidation of ammonium to nitrite. AOB belong to beta- and gammaproteobacteria. Betaproteobacteria AOB belong to two genera, *Nitrosomonas* and *Nitrosospira* and gamma-proteobacteria belong to the genus *Nitrosococcus* (Purkhold et al., 2000). In soil, the dominant AOB are *Nitrosospira* (Aigli et al., 2019; Purkhold et al., 2000; Purkhold et al., 2003). *Nitrosomonas* have been found in soil but dominate mainly in engineered ecosystems (Mobarry et al., 1996; Schramm et al., 1996), while *Nitrosococcus* are mainly found in marine environments (Campbell et al., 2011). Many studies have reported the isolation of AOM strains in the laboratory. Isolated AOB strains belong to *Nitrosomonas* and *Nitrosospira* genera and grow at neutral pH and optimal growth temperature at 25-30^{o}C (Winogradsky 1989, Head et al., 1993). However, the isolation of two acidophilic AOB strains that grow in acidic conditions was recently reported (Hayatsu et al., 2017; Picone et al., 2020).

In this project, only betaproteobacteria (*Nitrosospira multiformis* ATCC25196 and *Nitrosomonas. communis* Nm 2) were used in the *in vitro* bioassays for the activity of NIs. These two AOB strains are gram negative bacteria and were isolated from soil. AOB can derive all their energy and reductant for their growth from the oxidation of ammonia to nitrite as they encode all the enzymes participated in nitrification (ammonia monoxygenase complex (AMO), hydroxylamine oxidoreductase complex (HAO)) with each strain containing different copy numbers of these functional genes (Chain et. al., 2003; Norton et. al., 2008; Rice et. al., 2016; Kozlowski et. al., 2016; Sedlacek et. al., 2019). *N. multiformis* in contrast to *N. communis,* uses urea as an alternative N source.

Both strains are grown aerobically *N. multiformis* is routinely grown at 25-30°C in Skinner and Walker's medium (Skinner and Walker, 1961) containing 1 mM NH_4^+ [$(NH4)_2SO_4$] and phenol red (0.5 mg L^{-1}) as a pH indicator. *N. communis*, is routinely grown at 25-30°C in 1583 medium with 10 mM NH_4^+ (NH_4Cl) and phenol red (0.5 mg L^{-1}) as a pH indicator. Both strains are also grown in HK medium containing up to 30 mM NH_4^+ [$(NH_4)_2SO_4$] and phenol red (0.5 mg L^{-1}) as a pH indicator.

2.1.1.1 Skinner and Walker (SW) Medium

A. Introduction and Objective

The Skinner and Walker (SW) medium is designed for cultivating AOB and is used to assess their growth and activity by following nitrite production. This protocol outlines the preparation of SW medium, inoculation, and incubation conditions to promote AOB growth.

B. Materials and Equipment

B.1. Glassware and Equipment

- Glass flasks (e.g., "Duran" media bottles)
- 96-well plates
- Stirrer
- Autoclave
- pH meter
- 96-well plate reader
- (Shaking) Incubator

B.2. Media and solution preparation

- **Skinner and Walker medium (SW medium)**

Reagents	Amount	Units	Concentration
$(NH_4)_2SO_4$	0.066	g	1 mM NH_4^+
KH_2PO_4	0.2	g	1.47 mM
$CaCl_2$ $2H_2O$	0.04	g	0.27 mM
$MgSO_4$ $7H_2O$	0.04	g	0,16 mM
FeNaEDTA (7.5 mM)	1	ml	7.5 µM
Phenol red (0.05%)	1	ml	0.00005%
HEPES buffer (1M)	1	ml	1mM
5% Na_2CO_3	few drops		
Distilled water	Up to 1000	ml	

- **HEPES Buffer Preparation**
  A 1 M HEPES buffer is prepared by dissolving 14.298 g of HEPES and 2.4 g of NaOH granules in 60 ml of distilled water. The solution is then autoclaved for sterilization.

- **5% Sodium Carbonate Solution Preparation**
  A 5% Na_2CO_3 solution is prepared by dissolving 9.45 g of $Na_2CO_3 \cdot 10H_2O$ in 70 ml of distilled water. The solution is then autoclaved for sterilization.

C. Procedure

1. Dissolve the reagents in 1 L of deionised water. Stir the medium, autoclave, and cool to room temperature.
2. Adjust the pH by adding 1/1000th sterile HEPES buffer (1M) and neutralize with autoclaved 5% Na_2CO_3. A light pink colour indicates a pH of 7.5-8.0.
3. Store the medium at 4°C for future use.
4. Inoculate with a 1% (v/v) growing culture. Add a drop (approximately 50 µl) to an LB or R2A agar plate to check for contamination.
5. Incubate at 28°C in the dark, with or without shaking (see Part F: Special Considerations). Regularly neutralize the pH with Na_2CO_3 as needed.
6. Check growth by measuring nitrite concentration colorimetrically using the Griess test in a 96-well plate with diazotizing and coupling reagents (Shinn, 1941). For each measurement, triplicate standards ranging from 0 to 200 µM $NaNO_2$ are used, and absorbance is recorded at 540 nm.

D. Expected Results

- Production of nitrite and a colour change in the culture from pink to yellow, indicating acidification. In this case, the addition of 5% Na_2CO_3 is required, as described above.
- Measurement of nitrite concentration provides insight into AOB growth and activity.

E. Troubleshooting and Notes

- If precipitation occurs, verify reagent freshness and thorough mixing.
- Add Na_2CO_3 gradually to maintain pH and observe colour changes.

- Inoculate a new culture when nitrite concentrations exceed 200 µM, typically after a few days of growth.
- The medium and additional solutions can be stored at 4°C for months.

F. Special Considerations

- **Copper Deficiency:** The SW medium lacks copper, which is crucial for the activity of AMO, as copper facilitates electron transfer and catalysis during ammonia oxidation. Consider this when interpreting results related to enzyme function.
- **Cultivation under shaking:** AOB are routinely grown in the dark, without shaking. However, AOB are obligate aerobes, requiring oxygen for their metabolic processes, and gentle shaking (60-80 rpm) during cultivation can enhance their growth by increasing oxygen availability.

2.1.1.2. HK Medium

A. Introduction and Objective

The HK medium supports AOB cultivation and monitors nitrite production. This protocol details the steps to prepare HK medium, inoculate, and incubate AOB cultures for optimal growth and activity.

B. Materials and Equipment

B.1. Equipment

- Glass flasks (e.g., “Duran” media bottles)
- Stirrer
- Autoclave
- pH meter
- 96-well plate reader
- (Shaking) Incubator

B.2. Media and solution preparation

☐ **HK medium**

Reagents	Amount	Unit	Concentration
$(NH_4)_2SO_4$	0.66	g	10 mM NH_4^+
$CaCl_2$ $2H_2O$ 1M	1	ml	1 mM
$MgSO_4$ $7H_2O$ 1M	0.2	ml	0.2 mM
KCl 1M	1	ml	1 mM
phenol red (0.05%)	1	ml	0.00005%
Trace element solution	1	ml	0.001%
HEPES	4.24	g	15 mM
Distilled water	to 1000	ml	

- **Trace elements**

Compound	Amount	Unit
Na_2-EDTA	4292	mg

Compound	Amount	Unit
$MnCl_2$ x 2 H_2O	81	mg
H_3BO_3	62	mg
$ZnCl_2$	68	mg
Na_2MoO_4 x 2 H_2O	24	mg
$CoCl_2$ x $6H_2O$	24	mg
$NiCl_2$ x $6H_2O$	24	mg
$CuCl_2$ x $2H_2O$	17	mg
$FeCl_2$ x 4 H_2O	1988	mg
Distilled water	to 1000	ml

C. Procedure

1. Prepare the trace element solution in 1000 ml deionised water. The solution is then filtered for sterilization using 0.2 µm PES filters.
2. Dissolve reagents for HK medium in 1 L deionised water. Stir and adjust pH to 7.3 with 10% Na_2CO_3 for a pink colour. Autoclave and cool to room temperature.
3. Add 0.4 ml of 1 M KH_2PO_4, which will adjust the pH to 7.5.
4. Inoculate with 1% (v/v) growing culture. Test for contamination with a drop on LB or R2A agar plates.
5. Incubate cultures at 28°C in the dark, with/without shaking.
6. Regularly monitor pH through visual observation of the colour change and neutralize with Na_2CO_3 as needed. Check growth by nitrite concentration using the Griess test (Shinn, 1941).

D. Expected Results

As AOB grow, the medium turns from pink to yellow, and nitrite concentration increases, indicating successful cultivation.

E. Troubleshooting and Notes

- If precipitation occurs post-autoclaving, remake the medium.
- Carefully adjust Na_2CO_3 additions to maintain a stable pH.

F. Special Considerations

- AOB grow faster in HK medium, potentially due to the inclusion of copper at a standard concentration, and the improved pH stability provided by the higher HEPES concentration.

2.1.1.3. 1583 Medium

A. Introduction and Objective

This protocol describes the preparation of a culture medium specifically designed for AOB isolated from soil, based on a formulation from DSMZ (DSMZ n.d.). The medium supports optimal growth and activity by providing essential nutrients and maintaining pH stability.

B. Materials and Equipment

B.1. Equipment

- Glass flasks (e.g., “Duran” media bottles)
- Stirrer

- Autoclave
- pH meter
- 96-well plate reader
- (Shaking) Incubator

B.2. Media and solution preparation

☐ 1583 Medium [DSMZ (n.d.)]

Reagent	Amount	Unit	Concentration
NH_4Cl	535	mg	10 mM NH4+
KH_2PO_4	54	mg	0.4 mM
KCl	74	mg	1 mM
$MgSO_4 \cdot 7H_2O$	49	mg	0.2 mM
$CaCl_2 \cdot 2H_2O$	147	mg	1 mM
NaCl	584	mg	10 mM
Trace element solution	1	ml	0.001%
Cresol red solution	2	ml	0.0001%
Distilled water	to 1000	ml	

- **Trace Element Solution**

Reagent	Amount	Unit
Distilled water	975	ml
HCl, 1M	25	ml
$MnSO_4 \cdot 4H_2O$	45	mg
H_3BO_3	49	mg
$ZnSO_4 \cdot 7H_2O$	43	mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	37	mg
$FeSO_4 \cdot 7H_2O$	973	mg
$CuSO_4 \cdot 5H_2O$	25	mg

- **Cresol Red Solution**

Reagent	Amount	Unit
Cresol red	50	mg
Distilled water	to 100	ml

C. Procedure

1. Prepare Solution A by dissolving the specified amounts of each reagent in distilled water, adjusting the total volume to 1000 ml.
2. Stir the medium until fully dissolved and ensure no visible precipitate forms.
3. Autoclave the solution to sterilize it.
4. After autoclaving, allow the medium to cool to room temperature. The medium should be free of precipitates and have a yellow colour, indicating an acidic pH.
5. For pH maintenance use 1/1000th sterile HEPES buffer (1 M) dissolved in 0.6 M NaOH (14.298 g HEPES and 2.4 g NaOH granules in 60 ml distilled water).
6. Then adjust the pH of the medium to approximately 7.8 by adding sterile 10% $NaHCO_3$.
7. During the growth of the culture, monitor the pH closely. If the pH indicator changes to yellow, add $NaHCO_3$ to readjust the pH immediately.
8. Adjust aeration according to the density and activity of the culture.
9. Inoculate the fresh medium with a transfer of 1% (v/v) growing culture into glass flasks (e.g., Duran bottles).
10. Growth can be monitored by measuring the increase in nitrite concentration using the colorimetric Griess test. For this, nitrite concentrations in the culture are determined in a 96-well plate using diazotizing and coupling reagents. Duplicate standards between 0 and 200 µM $NaNO_2$ should be used, and absorbance is recorded at 540 nm.

D. Expected Results

The prepared medium should support the growth of AOB, allowing for the assessment of nitrite production and other metabolic activities.

E. Troubleshooting and Notes

- **pH management:** Regularly check and adjust the pH of growing cultures to ensure optimal growth conditions. For stock cultures, you can add either 5 g L^{-1} of $CaCO_3$ (which will remain as a precipitate) or 4.8 g L^{-1} of HEPES to maintain a neutral pH for extended period.
- **pH adjustment:** Phenol red can be used as an alternative to cresol red solution.
- **Aeration sensitivity:** Be cautious with aeration levels, particularly with low cell density cultures, to avoid damage to the bacteria. Aeration must be adjusted according to the density and activity of the culture. At low cell concentrations (which is the case most of the time), cells are sensitive to strong aeration (don't shake the cultures).
- **Inoculation:** Inoculate a new culture when nitrite concentrations exceed 200 µM, typically after a few days of growth.
- **Contamination:** To check for contamination by heterotrophic bacteria, add approximately 50 µl of the grown culture to an LB or R2A agar plate and incubate for observation.

2.1.2 Cultivation Protocols for Ammonia Oxidizing Archaea (AOA)

The second microbial group involved in the first nitrification step is the ammonia oxidizing archaea (AOA). AOA belong to the class *Nitrososphaeria* within the phylum Thaumarchaeota, and known AOA diversity is represented by four phylogenetic lineages: *Nitrososphaerales, Nitrosopumilales*, *Ca.* Nitrosotaleales, and the thermophilic *Ca.* Nitrosocaldales (Brochier-Armanet et al., 2008; Kerou et al., 2016), with *Nitrososphaerales* and *Nitrosotaleales* being the two dominant lineages in soil ecosystems. AOA are ubiquitous and highly diverse in terrestrial and marine ecosystems (Gubry-Rangin et al., 2010; Alves et al., 2018).

AOA dominate ammonia oxidation in acidic soils, which constitute about 30% of soil globally (Zhao et al., 2020). This group includes both acidophilic and neutrophilic isolates, which grow in liquid

cultures at an optimal temperature range of 30–42°C and belong to *Nitrososphaerales*. Acidophilic AOA, specifically those of the *Nitrosotalea* genus, grow in acidic liquid media with a pH range of 4–6 (Lehtovirta-Morley et al., 2014; Lehtovirta-Morley et al., 2011). For NI activity bioassays, both acidophilic (*N. sinensis* Nd2) and neutrophilic (*Ca.* N. franklandianus C13, *N. viennensis* EN76) AOA strains were used. These strains, isolated from soil, derive their energy from ammonia oxidation and encode the AMO complex. However, information on the oxidation of hydroxylamine to NO_2^- is limited (Lehtovirta-Morley et al., 2011; Lehtovirta-Morley et al., 2014; Tourna et al., 2011). The AMO enzyme in AOA is more complex than in AOB, as it contains three additional subunits with an unknown function (Hodgskiss et al., 2023).

All AOA strains are routinely grown aerobically in the dark with or without shaking. *Ca.* N. franklandianus C13 is incubated at 35-42°C in media supplemented with 2 mM NH_4^+ (NH_4Cl) and *N. sinensis* Nd2 at 28-35°with 0.5 mM NH_4^+. The former is cultured in HEPES-buffered modified freshwater medium (pH 7.5; Lehtovirta-Morley et al., 2014), while the latter is grown in MES-buffered freshwater medium (pH 5.2; Lehtovirta-Morley et al., 2011). *N. viennensis* EN76 is routinely grown at 35°C in HEPES-buffered freshwater medium (pH 7.5) with 2 mM NH_4^+ (NH_4Cl), with an optimal growth temperature of 42°C (Tourna et al., 2011).

2.1.2.1 Cultivation protocol for Nitrosotalea sinensis Nd2

A. Introduction and Objective

This protocol describes the preparation of Fresh Water (FW) medium and inoculation and incubation methods to support the growth of the AOA strain *N. sinensis*, allowing nitrite production to be assessed as an indicator of microbial activity.

B. Materials and Equipment

B.1. Glassware and Equipment

- Glass flasks (e.g., Duran media bottles)
- LB or R2A agar plates
- 0.2 µm bottle-top filters
- Vacuum pump
- pH meter
- 96-well plate reader
- (Shaking) Incubator

B.2. Media and Solution Preparation

☐ **Fresh Water Medium (FW Medium)**

Reagents	Amount	Concentration
Basal Salts (10x)	100 ml	1X
$NaHCO_3$ (1 M)	2 ml	2 mM
FeNaEDTA (7.5 mM)	1 ml	7.5 µM
Modified Trace Elements	1 ml	
NH_4Cl (1 M)	0.5 ml	0.5mM

Reagents	Amount	Concentration
MES hydrate	1.95 g	10 mM
Distilled water	to 1000 ml	

1. Dissolve all reagents in 900 ml of distilled water, then add additional distilled water to bring the total volume up to 1 L.
2. Adjust the pH to 5.0-5.3 at room temperature.
3. Sterilize the medium by filtering it through a 0.2 µm PES bottle-top filter.
4. Store the medium at 4°C for long-term use.

- **Basal Salts (10x)**

Reagents	Amount	Concentration
NaCl	10 g	171.1 mM
$MgCl_2 \cdot 6H_2O$	4 g	19.68 mM
$CaCl_2 \cdot 2H_2O$	1 g	6.8 mM
KH_2PO_4	2 g	14.7 mM
KCl	5 g	67.1 mM
Distilled water	to 1000 ml	

1. Dissolve all reagents in distilled water to a final volume of 1 L.
2. Stir thoroughly, autoclave, and allow to cool to room temperature. Ensure the solution is free of precipitates.

- **Trace Elements Solution**

Reagents	Amount	oncentratio
HCl (12.5 M)	8 ml	0.1 M
$MnCl_2 \cdot 4H_2O$	0.1 g	0.5 mM
H_3BO_3	0.03 g	0.5 mM
$ZnSO_4 \cdot 7H_2O$	0.144 g	0.5 mM
Na_2MoO_4 $2H_2O$	0.036 g	0.029 mM
$CoCl_2 \cdot 6H_2O$	0.19 g	0.8 mM
$NiCl_2 \cdot 6H_2O$	0.024 g	0.1 mM
$CuCl_2 \cdot 2H_2O$	0.002 g	0.012 mM
Distilled water	to 1000 mL	

1. Dissolve the reagents in distilled water to a final volume of 1 L and mix thoroughly.

C. Procedure

1. Inoculate fresh medium by adding 2% (v/v) of a logarithmic-phase culture into a glass flask.
2. Initiate a new culture when nitrite production exceeds 20 µM, typically after a few days.

3. To check for contamination, place a drop (approximately 50 µl) of the culture onto an LB or R2A agar plate.
4. Incubate the inoculated cultures in the dark at 35°C without shaking.
5. Monitor growth by measuring nitrite concentration, using a colorimetric assay (Griess test) in a 96-well plate with standards ranging from 0 to 200 µM $NaNO_2$. Record absorbance at 540 nm.

D. Expected Results

- Successful nitrite production indicated by colorimetric changes.
- Increase in nitrite concentration corresponding to microbial growth.

E. Troubleshooting and Notes

- The optimal growth temperature for *N. sinensis* Nd2 is 35°C, which is suitable for conducting *in vitro* assays. Cultivating the strain at slightly lower temperatures can slow down its growth rate, which is beneficial for routine laboratory maintenance as it reduces the frequency of subculturing, saving both time and materials.
- Ensure complete dissolution of salts to prevent precipitation.
- *N. sinensis* Nd2 cannot consume the entire substrate, as progressive nitrite accumulation inhibits its activity, leading to incomplete ammonia oxidation.

2.1.2.2. Cultivation protocol for Ca. Nitrosocosmicus franklandianus C13

A. Introduction and Objective

This protocol provides detailed instructions for preparing Modified Fresh Water (MFW) medium and related solutions, inoculation, and incubation conditions to cultivate *Ca*. Nitrosocosmicus franklandianus.

B. Materials and Equipment

B.1. Glassware and Equipment

- Glass flasks (e.g., Duran bottles, or 30 mL polystyrene flasks, or glass Schott bottles if scaling up. When using Schott bottles, fill only to half the volume (e.g., 500 mL in a 1 L bottle) with standard caps or grey rubber stoppers (CLS-4209-B45 stopper, anaerobic flange, GL45, grey bromobutyl rubber).
- Stirring device
- Autoclave
- 0.2 µm bottle-top filter
- Vacuum pump
- 96-well plate and plate reader
- pH meter
- (Shaking) Incubator

B.2. Media and Solution Preparation

Modified Fresh Water Medium (MFW Medium)

To prepare 1 L of MFW medium, dissolve the following in 900 mL of distilled water. Adjust pH to 7.5–7.6, then bring volume to 1 L and filter-sterilize (0.2 µm).

Reagent	Volume	Concentration
Basal Salts (10x)	100 mL	1x
$NaHCO_3$ (1 M)	2 mL	2 mM
FeNaEDTA (7.5 mM)	1 mL	7.5 µM
Modified Trace Elements	1 mL	
NH_4Cl (1 M)	2 mL	2 mM
HEPES buffer (1 M)	10 mL	10 mM
Modified Vitamin Solution	1 mL	
Selenite-Tungstate Solution	0.1 mL	
Distilled water	to 1000mL	

- **Basal Salts Solution (10x)**

Prepare a 10x basal salts solution by dissolving the following in up to 1 L of distilled water. Stir, autoclave, and cool to room temperature.

Reagent	Amount	Concentration
NaCl	10 g	171.1 mM
$MgCl_2 \cdot 6H_2O$	4 g	19.68 mM
$CaCl_2 \cdot 2H_2O$	1 g	6.8 mM
KH_2PO_4	2 g	14.7 mM
KCl	5 g	67.1 mM
Distilled water	to 1000 mL	

- **Trace Elements Solution**

Dissolve the following in up to 1L of distilled water:

Reagent	Amount	Concentration
HCl (12.5 M)	8 mL	0.1 M
$MnCl_2 \cdot 4H_2O$	0.1 g	0.5 mM
H_3BO_3	0.03 g	0.5 mM
$ZnSO_4 \cdot 7H_2O$	0.144 g	0.5 mM
Na_2MoO_4 $2H_2O$	0.036 g	0.029 mM
$CoCl_2 \cdot 6H_2O$	0.19 g	0.8 mM
$NiCl_2 \cdot 6H_2O$	0.024 g	0.1 mM
$CuCl_2 \cdot 2H_2O$	0.002 g	0.012 mM
Distilled water	to 1000 mL	

- **Selenite-Tungstate Solution**

Dissolve the following in 100 mL of distilled water, then autoclave. Some turbidity may form but does not affect usability.

Reagent	Amount	Concentration
NaOH	0.4 g	0.1 M
$Na_2SeO_3 \cdot 5H_2O$	0.006 g	0.23 mM
$Na_2WO_4 \cdot 2H_2O$	0.008 g	0.24 mM
Distilled water	to 100 mL	

- **Modified Vitamin Solution**
  Dissolve the following in 1 L of distilled water. Adjust pH to 7 with KOH and sterilize by filtration (0.2 µm filter).

Reagent	Amount	Concentration
Biotin	0.02 g	0.08 mM
Pyridoxine HCl	0.05 g	0.25 mM
Thiamine HCl	0.05 g	0.15 mM
Nicotinic acid	0.05 g	0.4 mM
Calcium pantothenate	0.05 g	0.2 mM
Vitamin B_{12}	0.01 g	7.4 µM
p-Aminobenzoic acid	0.05 g	0,36 mM
Distilled water	to 1000 mL	

- **HEPES Buffer (1 M)**

Dissolve 14.298 g of HEPES and 2.4 g of NaOH in 60 mL of distilled water. Autoclave and store at 4°C.

C. Procedure

1. Prepare FW Medium by dissolving all reagents in 900 mL of distilled water. Adjust the pH to 7.5–7.6, fill to a final volume of 1 L with distilled water, and sterilize by filtration (0.2 µm, PES).
2. Inoculate with 2% (v/v) culture at the logarithmic phase into a glass flask.
3. Place a drop (50 µL) of the grown culture onto LB or R2A agar plates to confirm no contamination.
4. Incubate at 35°C in the dark without shaking (see Part F: Special Considerations).
5. Monitor nitrite production using a Griess test. Prepare standards from 0 to 200 µM $NaNO_2$ and measure absorbance at 540 nm.
6. For cryo-preservation, grow cultures with 1 mM pyruvate, aliquot into 1.5 mL tubes, and freeze at -70°C. To revive, thaw at room temperature and inoculate with the entire 1 mL.

D. Expected Results

- Successful nitrite production indicates *Ca.* Nitrosocosmicus franklandianus growth.
- Nitrite level should reach >200 µM within several days.
- Generation Time: ~20 - 24 hours.

E. Troubleshooting and Notes

- Ensure no contamination by regular plate checks.
- If precipitates appear, check reagent freshness and solution clarity.
- Ensure that correct stoppers (grey rubber) are used with glass containers, as other stopper types inhibit growth.

- Microbial flocs of the organism are common and often visible

E. Special Considerations

- The optimal growth temperature for *Ca.* Nitrosocosmicus franklandianus C13 is 40°C, making it well-suited for *in vitro* assays. However, cultivating the strain at lower temperatures offers practical benefits for routine laboratory maintenance, such as slower growth rates that reduce the frequency of subculturing and help conserve time and resources.
- Not all vitamins are necessary; the organism can also grow with vitamin B1 as the sole supplement.
- *Ca.* N. franklandianus forms clumps that sediment at the bottom of containers, so mixing is recommended before sampling. During high-magnification microscopy, extracellular polymeric substances (EPS) may be observed around cell clumps.
- For optimum growth incubate at 40°C, under shaking at 80 rpm for cultures over 20 mL. Smaller cultures (20 mL or less) may be incubated without shaking.
- Transfer at ~1100-1200 µM nitrite concentration.
- Cultures can be harvested via filtration, followed by washing biomass off the filter.
- Previously known as *Candidatus* Nitrosocosmicus franklandus C13.

2.1.2.2. Cultivation protocol for Nitrososphaera viennensis EN76

A. Introduction and Objective

This protocol provides instructions for the preparation of Fresh Water Medium (FWM) and related solutions, inoculation, and incubation conditions to cultivate *Nitrososphaera viennensis*.

B. Materials and Equipment

B.1. Glassware and Equipment

- Glass flasks (e.g., Duran bottles, or 30 mL polystyrene flasks, or glass Schott bottles if scaling up. When using Schott bottles, fill only to half the volume (e.g., 500 mL in a 1 L bottle) with standard caps or grey rubber stoppers (CLS-4209-B45 stopper, anaerobic flange, GL45, grey bromobutyl rubber). Other stoppers inhibit growth.
- Stirring device
- Autoclave
- 0.2 µm bottle-top filter
- Vacuum pump
- 96-well plate and plate reader
- pH meter
- (Shaking) Incubator

B.2. Media and Solution Preparation

☐ **Fresh Water Medium (FWM)**

Prepare 1 L of FWM by dissolving the following in 900 mL of distilled water. Adjust pH to 7.5, then bring volume to 1 L and filter-sterilize (0.2 µm, PES).

Reagent	Volume	Concentration
Basal Salts (10x)	100 mL	1x
$NaHCO_3$ (1 M)	2 mL	2 mM

Reagent	Volume	Concentration
FeNaEDTA (7.5 mM)	1 mL	7.5 µM
Modified Trace Elements	1 mL	
NH_4Cl (1 M)	2 mL	2 mM
Sodium pyruvate (1 M)	1 mL	1 mM
HEPES buffer (1 M)	10 mL	10 mM
Distilled water	to 1000 mL	

- **Basal Salts Solution (10x)**

Prepare the basal salts solution by dissolving the following in 1 L of distilled water. Stir well, autoclave, and allow it to cool to room temperature.

Reagent	Amount	Concentration
NaCl	10 g	171.1 mM
$MgCl_2 \cdot 6H_2O$	4 g	19.68 mM
$CaCl_2 \cdot 2H_2O$	1 g	6.8 mM
KH_2PO_4	2 g	14.7 mM
KCl	5 g	67.1 mM
Distilled water	to 1000 mL	

- **Trace Elements Solution**

Dissolve the following in up to 1 L of distilled water:

Reagent	Amount	Concentration
HCl (12.5 M)	8 mL	0.1 M
$MnCl_2 \cdot 4H_2O$	0.1 g	0.5 mM
H_3BO_3	0.03 g	0.5 mM
$ZnSO_4 \cdot 7H_2O$	0.144 g	0.5 mM
Na_2MoO_4 $2H_2O$	0.036 g	0.15 mM
$CoCl_2 \cdot 6H_2O$	0.19 g	0.8 mM
$NiCl_2 \cdot 6H_2O$	0.024 g	0.1 mM
$CuCl_2 \cdot 2H_2O$	0.002 g	0.012 mM
Distilled water	to 1000 mL	

- **HEPES Buffer (1 M)**

Dissolve 14.298 g of HEPES and 2.4 g of NaOH in 60 mL of distilled water. Autoclave and store at 4°C.

C. Procedure

1. Prepare FWM by dissolving all reagents in 900 mL of distilled water. Adjust the pH to 7.5, fill up to 1 L with distilled water, and sterilize by filtration (0.2 µm, PES).

2. Inoculate the fresh medium with 0.05–10% (v/v) of stationary phase culture in glass flasks or polystyrene flasks.
3. Add a drop (approximately 50 µL) of the grown culture onto LB or R2A agar plates to verify absence of contamination.
4. Incubate at 35°C in the dark without shaking (see Part F: Special Considerations).
5. Monitor nitrite production by the Griess test. Prepare standards from 0 to 200 µM $NaNO_2$ and measure absorbance at 540 nm.
6. For cryo-preservation, grow cultures in FW medium with pyruvate for ROS detoxification, aliquot into 1.5 mL tubes, and freeze at -70°C. To revive, thaw at room temperature and inoculate with the entire 1 mL.

D. Expected Results

- Successful nitrite production indicates *N. viennensis* growth, with nitrite levels reaching >1000 µM within several days.
- Generation Time: ~12 hours.

E. Troubleshooting and Notes

- Ensure no contamination by regular plating on LB or R2A agar.
- Check for precipitates, which may indicate reagent degradation or inadequate mixing.
- Ensure correct shaking speed (80 rpm) for larger cultures and avoid specific plastics to maintain culture growth.

F. Special Considerations

- The optimal growth temperature for *N. viennensis* is 42°C, suitable for *in vitro* assays. Lower cultivation temperatures are preferred for routine laboratory maintenance to slow growth and reduce subculturing needs.
- *N. viennensis* cultures are very stable and can remain viable in the stationary phase for several days. Ideally, transfer at ~1100–1200 µM nitrite concentration**.**
- For optimum growth incubate at 42°C, under shaking at 80 rpm for cultures over 20 mL.
- Suggested method for harvesting is centrifugation due to cell adherence issues on filters.
- Pyruvate addition serves as ROS detox and is not used as a heterotrophic nutrient.
- Catalase can be added to alleviate ROS at 5–50 U/mL; higher concentrations may cause floc formation.
- Avoid using certain plastics that may affect growth; polystyrene has been observed to be suitable.
- If harvesting cells for further cultivation, concentration by centrifugation is recommended over filtration as cells are difficult to remove from filters.

2.1.3. Nitrite Assays Using a 96-Well Plate Format for Liquid Cultures

2.1.3.1. Nitrite assay for liquid cultures with detection range 0-200 µM

A. Introduction and Objective

This protocol details a colorimetric assay to measure nitrite concentration in liquid culture samples using a 96-well plate format. The assay quantifies nitrite levels based on a diazotization reaction and is applicable to studies of microbial nitrogen cycling.

B. Materials and Equipment

B1. Reagents and Solutions

- **Diazotizing Solution (Reagent 1):** Dissolve 5.0 g sulfanilamide in 100 mL of 3.3 M HCl. To prepare of 3.3 M HCl, mix 27.5 mL HCl (37%, 12M) with 72.5 mL dH_2O. Store at 4°C for up to 2 months.
- **Coupling Reagent (Reagent 2):** Dissolve 0.30 g NED (N-(1-naphthyl)-ethylenediamine dihydrochloride) in 100 mL of 0.12 M HCl. To prepare 0.12 M HCl, mix 1 mL HCl (37%, 12M) with 99 mL dH_2O. Store at 4°C; discard if the solution turns brown.
- **Stock Nitrite Solution (0.1 M $NaNO_2$):** Dissolve 1.725 g $NaNO_2$ in 250 mL dH_2O to prepare a 0.1M solution. For a 200 µM working standard, mix 20 µL of 0.1 M $NaNO_2$ with 9.98 mL dH_2O.

C. Procedure

1. Sample Preparation: Add 100 µL of each liquid culture sample to designated wells.

2. Reagent Addition: To each well, add 20 µL of Diazotizing Solution (Reagent 1) and 20 µL of Coupling Reagent (Reagent 2). Add diazotizing solution first, then the coupling reagent.
3. Incubation and Measurement: Incubate the plate for 10 minutes at room temperature. Measure absorbance at 540 nm to determine nitrite concentration in each well.
4. Nitrite Standard Preparation: Prepare a nitrite standard series (0–200 µM) in a 96-well plate using dH_2O as the matrix. In column A, add 100 µL of 200 µM nitrite solution to well A1. In wells A2–A8, add 100 µL dH_2O. Serially dilute by transferring 100 µL from well A1 to A2, mix well, then transfer 100 µL from A2 to A3, continuing down to well A8, creating concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.13, and 0 µM nitrite.

E. Expected Results
The absorbance readings at 540 nm should correlate linearly with nitrite concentration in the standard series. Use the standard curve to calculate the nitrite concentration in each sample.

F. Troubleshooting and Notes
- Ensure that both reagents are fresh; replace any solution that shows discoloration.
- Use only distilled water as the solvent for accurate results in liquid cultures.

2.1.3.2. Alternative Nitrite assay for liquid cultures with detection range 0-1000 µM

A. Introduction and Objective
This alternative protocol provides an extended range assay for measuring nitrite concentration in liquid cultures, enabling direct measurement without requiring initial sample dilution. This method, based on colorimetric analysis, is suitable for cultures with higher nitrite concentrations (100–1000 µM), though it may be less suitable for strains with lower nitrite consumption rates, such as *N. sinensis* Nd2.

B. Materials and Equipment

B.1. Reagents and Solutions

1. **Color Reagent**: Prepare by dissolving 5.18 mM sodium salicylate, 2.15 x 10^{-5} M sodium nitroprusside, and 0.1 M NaOH in dH_2O.
2. **Oxidation Solution**: Prepare by dissolving 3.91 x 10^{-5} M of dichloroisocyanuric acid in dH_2O.
3. **Sulfanilamide/NED Reagent**: Dissolve 0.058 M sulfanilamide, 1.9 mM N-(1-naphthyl)-ethylenediamine dihydrochloride, and 2.22 M ortho-phosphoric acid (85%) in dH_2O. Prepare fresh as needed to ensure reagent quality.

C. Procedure

1. **Sample Preparation**: Add 20 µL of each culture sample to a tube. Add 780 µL growth medium to each sample tube.
2. **Reagent Addition**: Add 200 µL of sulfanilamide/NED reagent to each tube containing the culture sample and growth medium. Mix thoroughly by gently pipetting up and down.
3. **Incubation**: Incubate the samples in the dark at room temperature for 30 minutes to allow complete color development.
4. **Measurement**: Transfer 200 µL of each reaction mixture to a 96-well plate. Measure absorbance at 545 nm using a UV spectrophotometer.
5. **Standard Curve and Quantification**: Generate a nitrite standard curve with concentrations ranging from 100 to 1000 µM to ensure accurate quantification within this broader range. Use this standard curve to determine the nitrite concentration in each sample directly.

D. Expected Results

Absorbance readings at 545 nm should correlate linearly with nitrite concentration in the 100–1000 µM range. Calculate sample concentrations using the standard curve prepared under identical conditions.

F. Special Considerations

- This method allows direct measurement in samples without dilution, which is beneficial for cultures with high nitrite production.
- For strains with lower nitrite production or slower substrate utilization (e.g., *N. sinensis* Nd2), the assay may yield suboptimal sensitivity.
- Ensure that reagents are fresh and colourless before use, as any discoloration may affect assay accuracy.

2.2. Omics Protocols

This module outlines the protocols for RNA extraction, in our approach to characterizing the physiological effect of NIs at the cellular level in specific AOM. The primary aim is to identify the cellular responses to NIs in targeted strains of AOB and AOA, including *N. multiformis*, *N. communis*, *N. viennensis*, *N. sinensis*, and *Ca.* N. franklandianus.

Within this project, we employ a dual-omics approach—proteomics and transcriptomics—to detect NI-induced changes in protein and RNA expression levels in AOM strains. Following the cultivation protocols in Module 2.1, each strain is exposed to selected synthetic and biological NIs at the onset of exponential growth to assess NI effects on cellular processes, with NI-free cultures serving as controls. To monitor NI activity, batch cultures are regularly sampled to measure nitrite production, which serves as a proxy for AOM activity. At specific time points, samples are collected for detailed proteomic and transcriptomic analyses, allowing us to map the molecular pathways impacted by NI treatment.

This module provides detailed protocols for the extraction of RNA from collected biomass, supporting downstream analyses that will help elucidate the mechanisms underlying NI effects on AOA strains. This section will be expanded to include protocols for RNA and protein extraction for AOA and AOB as these methods are further developed and validated.

2.2.1. RNA Extraction

A. Objective

To extract RNA and proteins from collected biomass

B. Materials and Equipment

Item	Purpose
mirVana™ miRNA Isolation Kit	RNA extraction
MCE Membrane Filter (0.2 µm)	Biomass filtration
Falcon Tubes (50 mL)	Filter storage
RNase/DNase-free tubes (1.5 mL)	Sample processing
Matrix B Bead Beating Tube	For *Ca.* N. franklandianus lysis
Acid Phenol	Phase separation
Chloroform	Double extraction phase
EtOH (100%)	RNA precipitation
NanoPhotometer N60	RNA quantification

2.2.1.1. Cell Harvesting for Transcriptomics

A. Objective

This protocol describes the filtering of AOM biomass onto small filters for RNA extraction. For larger amounts or when concentrating for further cultivation, larger filters can be used with a vacuum pressure bottle top apparatus, recommended for filtering over 250-300 mL of culture.

B. Procedure

1. Prepare a sterile filter apparatus with a 45 mm MCE filter attached to the top of a Schott bottle.
2. Add medium to filter apparatus and use a vacuum pump to filter medium and attach cells to the MCE filter.
3. Using sterile tweezers, remove the filter and place in a sterile 50 mL falcon tube and immediately put on ice.
4. Freeze filters at -70°C until further cell lysis and preparations.

C. Special Considerations

- Filters should be frozen as soon as possible but can be transported of the freezer after 3-5 replicates have been filtered.

2.2.1.2. RNA extraction with mirVana™ miRNA Isolation Kit for AOA

A. Solutions

- Lysis/Binding Buffer
- Homogenate Additive
- Acid Phenol-Chloroform
- 100% Ethanol
- miRNA Wash Solution 1
- miRNA Wash Solution 2/3
- DEPC (Diethylpyrocarbonate) ddH_2O
- 3 M Sodium Acetate
- Glycogen
- 70% Ethanol

B. Procedure

1. Resuspend the filter in 600-700 µL of Lysis/Binding Buffer using 1 ml syringe with needle. Resuspend thoroughly with the syringe for better wash and lysis of the filter. 2 to 5 minutes (depending on the microorganism) are enough for washing or lysing the cells on the filter. Keep the samples on ice between different steps. If needed, wash cell off of filter and bead beat at a speed of 4 m/s for 30s to lyse cells. Cool for 5 min on ice.
2. Incubate for 10 minutes on ice.
3. Preheat DEPC ddH_2O at 95ºC for later use.
4. Transfer the mixture into an RNAse/DNAse-free 2 mL Eppendorf tube (Sarstedt) and add 60 µL of Homogenate Additive. Vortex the samples and incubate on ice for 10 minutes.
5. Add 600 µL of acid Phenol-Chloroform and vortex for 60 seconds (from this step, the entire protocol is performed in a fume hood)
6. Centrifuge at 10,000 x g for 5 minutes at room temperature.
7. Carefully remove the upper phase without touching the intermediate or lower phase (approximately 550 µL) and transfer it into a fresh 2 mL Eppendorf tube.
8. Store the lower phase (non-aqueous containing proteins) in ice until the protein extraction.
9. Add 700 µL of 100% Ethanol at room temperature and mix thoroughly with pipette or vortex for 30 seconds.
10. Pass the mixture through a filter into a cartridge supplied with the kit (700 µL can be passed at once; repeat this step). Centrifuge at 10,000 x g for 15 seconds and discard the flow-through.
11. Wash the filter with 700 µL of miRNA Wash Solution 1, centrifuge for 15 seconds at 10,000 x g and discard the flow-through.
12. Wash the filter with 500 µL of miRNA Wash Solution 2/3, centrifuge for 15 seconds at 10,000 x g and discard the flow-through.
13. Repeat the previous washing step.
14. Place the filter into a fresh cartridge and add 100 µL of 95°C DEPC ddH_2O. Incubate for 10 minutes at room temperature.
15. Centrifuge at 10,000 x g for 1 minute, discard the filter.
16. Let the RNA homogenise for 30 minutes in ice before measuring the concentration using a NanoDrop. Perform phenol cleanup if necessary.

2.2.1.3. RNA Sample Cleanup and DNA Digestion

A. Objective

To clean up RNA from phenol residues and digest DNA in the RNA samples.

B. Procedure

B1. Phenol Cleanup

1. Add 200 µL of DEPC ddH_2O to a total volume of 300 µL. Include 20 µL of 3 M sodium acetate and 1 µL of glycogen, then vortex.
2. Add 550 µL of ice-cold 100% ethanol, vortex, and incubate at -80°C for 30 minutes or overnight at 4°C. Centrifuge at 11,000 x g for 20 minutes at 4°C. Discard the liquid, taking care not to touch the sides of the Eppendorf with the pipette tip (RNA is precipitated).
3. Add 1 mL of ice-cold 70% ethanol, centrifuge at 11,000 x g for 10 minutes at 4°C, and carefully remove the liquid with a pipette. Spin down quickly again.
4. Carefully remove any remaining liquid with a 10 µL tip and allow the tube to air dry for 10 minutes.

5. Resuspend RNA in 100 µL of DEPC ddH_2O and measure the concentration.

B2. Turbo DNase Digest

1. To the 100 µL RNA solution, add 11.2 µL of Turbo DNase Buffer and 1-2 µL of Turbo DNase. Use 1 µL (2 U) for 10 µg of RNA; if the RNA amount exceeds 10 µg, use 2 µL.
2. Incubate at 37°C for 1-2 hours for complete digestion of DNA.
3. Add 11.2 µL of Inactivation Reagent, flick the tube several times, and centrifuge at room temperature for 1.5 minutes at 11,000 x g.
4. Transfer the RNA into a new DNase/RNase-free 1.5 mL Eppendorf tube.
5. Measure the concentration using Qubit.
6. Perform a control PCR with AOM strain specific primers and run an agarose gel 1-2% to verify that there is no DNA in the samples.

Notes

- *Always work in a clean environment and use RNase/DNase-free consumables to prevent contamination.*
- *Follow safety guidelines, especially when handling hazardous chemicals like phenol and chloroform.*
- *Make sure to use the kit to extract total RNA rather than only microRNA. If protocol is followed as described here, total RNA should be extracted.*

2.3. Soil Microbial Activity and Abundance Assessment

This module presents protocols to assess the activity and abundance of key soil microorganisms, particularly targeting AOA, AOB, complete ammonia oxidizers (comammox), and nitrite oxidizers, in response to NIs. The goal is to evaluate how NIs affect the metabolic activity of these microorganisms, which play essential roles in soil nitrogen cycling.

Stable-isotope probing (SIP) is a core technique in this module, providing a functional assessment by linking microbial metabolic activity to specific taxa within complex soil communities. When coupled with downstream analyses, SIP allows us to trace the *in-situ* effects of NIs on the activity of targeted nitrifiers. Quantitative PCR (qPCR) protocols are also included to measure the abundance of target nitrifiers in soil samples, providing quantitative data on how NIs affect their population levels. In addition, protocols for the quantification of ammonium, nitrite, and nitrate levels in soil samples offer a direct assessment of nitrogen intermediates, which are critical for understanding the biochemical impact of NIs on soil nitrogen dynamics.

This module offers detailed SIP sample preparation steps, qPCR conditions for soil nitrifiers, and guidance for analysing NI-induced changes in microbial activity.

2.3.1. DNA Stable-Isotope Probing (DNA-SIP)

DNA Stable-Isotope Probing (DNA-SIP) is a method used to link microbial identity with function by incorporating isotopically labeled substrates (e.g., 13C or 15N) into the DNA of active microorganisms within a sample. By separating DNA based on density, DNA-SIP helps to distinguish microorganisms actively utilizing labelled substrates from others, thereby revealing functional microbial players within complex communities.

A. Materials and Equipment

A1. Consumables and Reagents

- Polyallomer tubes
- Cesium chloride (CsCl)
- TE buffer
- 70% ethanol
- 30% PEG6000/1.6 M NaCl solution (30 g PEG6000 and 9.35 g NaCl in 100 mL distilled H_2O, autoclave and store at room temperature)
- PCR-grade H_2O

A2. Equipment

- Refractometer
- Analytical balance
- Nanoprotected hood
- Syringe and needle (for adding CsCl)
- Soldering iron (250°C) and black "press" for sealing tubes
- Ultracentrifuge with MLN80 rotor
- Torque wrench
- Peristaltic pump
- Bio-Rad fractionator
- Centrifuge (16,000 g, with temperature control)
- Dog nail trimmer (for cutting tube necks)
- Microcentrifuge tubes (1.5 mL)

B. Procedure

B1. CsCl Preparation

1. Dissolve 1.27 g CsCl in 1 mL TE buffer (e.g., 127 g CsCl in 100 mL TE buffer) within the nanoprotected hood.
2. Allow solution to reach room temperature and adjust the refractive index to 1.4004 using small additions of TE buffer or CsCl.

   ***Note**: Calibrate refractometer with 25 µL H_2O before measuring.*

B2. Prepare Centrifuge Tubes

1. Label each polyallomer tube on two sides of the curved top surface.
2. Add ~8 mL CsCl solution, leaving space for the DNA sample. Use a syringe and needle for faster addition.
3. Eliminate bubbles by moving the large air pocket to "collect" bubbles on the sides.
4. Add DNA (1–2 µg for amplicon work or 6–8 µg for metagenomics). Ensure equivalent DNA solution volumes across samples for consistent CsCl dilution.
5. Top up with CsCl solution until the meniscus is just below the tube neck.
6. Weigh tubes in pairs, adjusting with CsCl solution to balance within 0.010 g.

B3. Seal Tubes and Centrifuge

1. Seal tubes with a 250°C soldering iron and the black "press." Turn off the soldering iron after use.
2. Insert the MLN80 rotor into the metal stability insert on the table.
3. Record tube positions, cap with red tops, and secure black seals with Teflon rings on top. Hand-tighten seals with a torque wrench set to 100 in/lb.
4. Carefully place the rotor in the centrifuge, set to 50,000 rpm for 72 hours at 25°C.

5. Start vacuum before initiating the spin. Monitor the centrifuge until it reaches full speed.

B4. Post-Centrifugation Tube Removal

1. After centrifugation, place the rotor in the stability insert. Loosen seals using the torque wrench. Be ready for the slight "jump" when releasing at 100 in/lb.

B5. Fractionation System Preparation

1. Autoclave tubes, pipes, water, and needle to ensure sterility for fractionation.

B6. Sample Fractionation

16. Verify the fractionation system is connected properly. Flow water through the system to confirm seal.
17. Trim the tube neck using a dog nail trimmer, leaving a small neck portion intact.
18. Place the sample tube securely in the stand and pierce with the needle.
19. Start the peristaltic pump. Use the Bio-Rad fractionator to collect ~325 µL per fraction into microcentrifuge tubes.
20. Use 25 µL of each fraction to measure the refractive index. For ^13C-labeled incubation, the refractive index should reach approximately 1.4040.
21. Expected fractionation time is ~45 minutes per tube.

B7. DNA Precipitation

22. Add two volumes of 30% PEG6000/1.6 M NaCl solution to each fraction, mix, and incubate overnight at 5°C.
23. Follow Griffith's protocol for DNA recovery: centrifuge at 16,000 g, 5°C, for 20 minutes; decant supernatant, add 1 mL of 70% ice-cold ethanol, mix, centrifuge again, and dry. Dissolve DNA in 50 µL PCR-grade H_2O.

    ***Note**: DNA pellets may not be visible. Carefully add water to the expected pellet location without disturbing the tube*.

B8. Visualization and Analysis

24. Run standard agarose gel electrophoresis on samples before qPCR to verify the distribution of labelled DNA across the buoyant density gradient.

C. Expected Results

The expected outcome is the successful separation of isotopically labeled DNA, which can then be analysed for microbial identity and function using downstream techniques like qPCR. This allows insight into the active microbial groups metabolizing the labeled substrate.

D. Troubleshooting and Notes

- **CsCl Adjustment**: Small adjustments to CsCl concentration may be needed to achieve the target refractive index.
- **Centrifugation Precaution**: Ensure seals are tight to prevent leaks under high pressure. Watch for consistent mass matching to avoid rotor imbalance.
- **DNA Visibility**: DNA pellets may be invisible; handle samples carefully to avoid disturbing or losing the pellet.

2.3.2. Extraction and Quantification of Ammonium, Nitrite, and Nitrate from Soil Samples

This protocol outlines the extraction and quantification of ammonium, nitrite and nitrate from soil samples using KCl solution as the extraction medium. Ammonium and nitrate levels serve as key indicators of soil nitrogen cycling, providing insights into microbial nitrogen transformation activities and the effects of treatments such as NIs. The protocol consists of two parts: (1) extraction of nitrogen species from soil using KCl, and (2) quantification of ammonium, nitrite, and nitrate in the extracted solution using a 96-well plate format. Ammonium (NH_4^+-N), nitrite (NO_2^--N) and nitrate (NO_3^- -N) in the extracts were determined using a modified indophenol method based on the Berthelot reaction (Kandeler and Gerber, 1988), and on the VCl3/Griess approach (Doane and Horwáth, 2003; Shin 1941), respectively, as cited in Hink et al. (2018).

A. Materials and Equipment

A1. Consumables:

- 15 mL falcon tubes
- 1.5 mL Eppendorf tubes
- 96-well microplate
- Parafilm or plastic wrap

A2. Reagents and Solutions

- 1 M KCl solution: Dissolve 74.55 g KCl in 1000 mL distilled water
- Diazotizing reagent (Reagent 1): 5.0 g sulfanilamide dissolved in 100 mL 3.3 M HCl
- Coupling reagent (Reagent 2): 0.3 g NED dissolved in 100 mL 0.12 M HCl
- Vanadium chloride solution (for nitrate assay only): 3.5 g VCl_3 in 50 mL 1 M HCl
- Stock nitrite solution: 0.1 M $NaNO_2$ in water
- Stock nitrate solution: 0.1 M $NaNO_3$ in water
- Colour reagent solution (for ammonium assay): Mix 0.3 M NaOH, sodium salicylate solution, and water (1:1:1 ratio)
- Oxidation solution (for ammonium assay): 50 mg sodium dichloroisocyanuric acid in 50 mL water
- Stock ammonium solution: 0.5 M NH_4Cl in water

A3. Equipment

- Centrifuge
- Vortex mixer
- Shaker
- Microplate reader for absorbance measurements

B. Procedure

B1. Soil Extraction of Ammonium, Nitrite, and Nitrate

1. Weigh 2 g of soil and transfer it into a 50 mL falcon tube.
2. Add 20 mL of 1 M KCl solution to each soil sample tube. Rotate the tubes for 30 minutes to allow complete extraction of nitrogen species.
3. Centrifuge tubes at 4000 rpm for 10 minutes at room temperature (or 4°C if samples were stored at lower temperatures). Transfer the supernatant into clean 15 mL falcon tubes (or 1.5 mL Eppendorf tubes if only small volumes are needed).

4. For short-term storage, place extracts in a refrigerator. For long-term storage, keep the samples at –20°C.

Note: *Nitrite can degrade at low pH in frozen samples.*

B2. Ammonium Assay

1. Prepare a standard series from 0 to 500 µM for ammonium (NH_4Cl) in a solvent-matching matrix (e.g., KCl solution).
2. In a 96-well plate, pipette 50 µL of each standard concentration, creating a dilution series from 500 µM to 0 µM.
3. Add 50 µL of each soil extract sample to designated wells.
4. To each well, add 50 µL of colour reagent solution (mix of NaOH and sodium salicylate). Add 20 µL of oxidation solution to each well.
5. Cover plate with parafilm and foil, then incubate for 30 minutes at room temperature with gentle shaking.
6. Measure absorbance at 660 nm.

B3. Nitrite and Nitrate Assay

1. Prepare a standard series from 0 to 200 µM for both nitrite ($NaNO_2$) and nitrate ($NaNO_3$) in a solvent-matching matrix (e.g., KCl solution). In a 96-well plate, pipette 100 µL of each standard concentration, creating a dilution series from 200 µM to 0 µM.
2. Add 100 µL of each soil extract sample to designated wells.
3. To each well, add 60 µL of diazotizing reagent (sulfanilamide solution) for soil KCl extracts. Immediately add 20 µL of coupling reagent (NED solution).
4. Measure absorbance at 540 nm to determine nitrite concentration.
5. For nitrate, add 20 µL of vanadium chloride solution to each well after nitrite measurement.
6. Cover plate and incubate at ~35°C for 90 minutes.
7. Measure absorbance at 540 nm (the nitrate concentration is calculated by subtracting the initial nitrite measurement from the total absorbance).

C. Expected Results

The assays should yield absorbance values that can be used to calculate the concentrations of ammonium, nitrite, and nitrate in each soil sample. Standard curves generated from known concentrations of ammonium, nitrite, and nitrate will enable quantification by comparison to sample absorbance values.

D. Troubleshooting and Notes

1. **Nitrite Instability in Low pH:**
- For acidic soils, nitrite may degrade at lower temperatures. Ensure soil pH is known and avoid freezing if pH is low.
2. **Reagent Stability:**
- The diazotizing solution and coupling reagent should be stored at 4°C.
- Prepare fresh vanadium chloride solution and oxidation solution for reliable results.
3. **Incubation Conditions for Nitrate Measurement:**
- Incubate nitrate assays at ~35°C to improve reaction completion. Adjust incubation times if needed.
4. **Matrix Matching for Standards:**
- Ensure the standards are prepared in the same matrix as the samples (KCl for soil extracts) to avoid discrepancies in absorbance measurements.

2.3.3. Quantitative PCR (qPCR) for soil nitrifiers

2.3.3.1. Quantitative PCR (qPCR) for AOB amoA gene

This qPCR protocol quantifies the *amoA* gene of AOB, encoding the alpha subunit of the AMO enzyme complex. Quantifying *amoA* in environmental samples helps assess the abundance of AOB populations, providing insights into ammonia oxidation dynamics. The qPCR is performed using the primers *amoA-1F* and *amoA-2R*, following the methods described by Rotthauwe et al. (1997).

A. Materials and Equipment

A1. Primers:

- *amoA-1F* Primer: 5'-GGGGTTTCTACTGGTGGT-3'
- *amoA-2R* Primer: 5'-CCCCTCKGSAAAGCCTTCTTC-3' [K = G or T; S = G or C]

A2. Reagents:

- KAPA SYBR FAST qPCR Master Mix (2×), Universal
- Bovine Serum Albumin (BSA, 20 mg mL^{-1})
- Template DNA
- Double distilled water (dd H_2O)

A3. Equipment:

- Bio-Rad CFX Connect Real-Time PCR System (or other Real-Time PCR System)
- Pipettes and filter tips
- Microcentrifuge tubes

B. Procedure

1. **Prepare the qPCR Reaction Mix:** In a 10 µL reaction volume, combine the following reagents:

Reagent	Volume (µL)	Final Concentration
KAPA SYBR FAST qPCR Master Mix (2×)	5	1×
amoA-1F Primer (20 pmol $µL^{-1}$)	0.1	0.2 µM
amoA-2R Primer (20 pmol $µL^{-1}$)	0.1	0.2 µM
BSA (20 mg mL^{-1})	0.2	0.1 mg mL^{-1}
DNA Template (1 ng $µL^{-1}$)	2	0.2 ng $µL^{-1}$
dd H_2O to 10 µL	2.6	

2. **Set Up Thermocycling Conditions:** Load the reaction tubes into the Real-Time PCR System and use the following cycling conditions:

Step	Temperature	Time	Cycle(s)
Initial Denaturation	95 °C	3 min	1 cycle
Denaturation	95 °C	5 sec	40 cycles
Annealing	57 °C	10 sec	
Extension	72 °C	30 sec	
Melting Curve Analysis	95 °C	1 min	1 cycle
	65–95 °C	sec/0.5°C increment	

3. **Melting Curve Analysis:** After amplification, perform a melting curve analysis from 65 °C to 95 °C with 0.5 °C increments every 5 seconds to confirm the specificity of the qPCR products.

2.3.3.2. Quantitative PCR (qPCR) for AOA amoA gene

This qPCR protocol quantifies the *amoA* gene of AOA for the assessment of the abundance of AOA populations, providing insights into ammonia oxidation dynamics. The qPCR is performed using the primers *Arch-amoAF* and *Arch-amoAR*, following the methods described by Francis et al. (2005).

A. Materials and Equipment

A1. Primers:

- *Arch-amoAF* Primer: 5'-STAATGGTCTGGCTTAGACG-3'
- *Arch-amoAR* Primer: 5'-GCGGCCATCCATCTGTATGT-3' [S = G or C]

A2. Reagents:

- KAPA SYBR FAST qPCR Master Mix (2×), Universal
- Bovine Serum Albumin (BSA, 20 mg mL^{-1})
- Template DNA
- Double distilled water (dd H_2O)

A3. Equipment:

- Bio-Rad CFX Connect Real-Time PCR System (or other Real-Time PCR System)
- Pipettes and filter tips
- Microcentrifuge tubes

B. Procedure

1. **Prepare the qPCR Reaction Mix:** In a 10 µL reaction volume, combine the following reagents:

Reagent	Volume (µL)	Final Concentration
KAPA SYBR FAST qPCR Master Mix (2×)	5	1×

Reagent	Volume (µL)	Final Concentration
Arch-amoAF Primer (20 pmol µL^{-1})	0.1	0.2 µM
Arch-amoAR Primer (20 pmol µL^{-1})	0.1	0.2 µM
BSA (20 mg mL^{-1})	0.2	0.1 mg mL^{-1}
DNA Template (1 ng µL^{-1})	2	0.2 ng µL^{-1}
dd H_2O to 10 µL	2.6	

2. **Set Up Thermocycling Conditions:** Load the reaction tubes into the Bio-Rad CFX Connect Real-Time PCR System and use the following cycling conditions:

Step	Temperature	Time	Cycle(s)
Initial Denaturation	95 °C	3 min	1 cycle
Denaturation	95 °C	15 sec	45 cycles
Annealing	53 °C	30 sec	
Extension	72 °C	45 sec	
Melting Curve Analysis	95 °C	1 min	1 cycle
	65–95 °C	5 sec/0.5°C increment	

3. **Melting Curve Analysis:** After amplification, perform a melting curve analysis from 65 °C to 95 °C with 0.5 °C increments every 5 seconds to confirm the specificity of the qPCR products.

2.3.3.2.1 Alternative Quantitative PCR (qPCR) for AOA amoA gene

This alternative qPCR protocol quantifies the *amoA* gene of AOA. The qPCR is performed using the primers *crenamoA23F* and *crenamoA616R*, following the methods described by Tourna et al., 2008.

A. Materials and Equipment

A1. Primers:

- *crenamoA23F* Primer: 5'- ATGGTCTGGCTWAGACG -3'
- *crenamoA616R* Primer: 5'- GCCATCCATCTGTATGTCCA -3'

A2. Reagents:

- QuantiNova 2X
- Template DNA

- Double distilled water (dd H_2O)

A3. Equipment:

- Bio-Rad CFX Connect Real-Time PCR System (or other Real-Time PCR System)
- Pipettes and filter tips
- Microcentrifuge tubes

B. Procedure

1. **Prepare the qPCR Reaction Mix:** In a 20 µL reaction volume, combine the following reagents:

Reagent	Volume (µL)	Final Concentration
QuantiNova 2X, Qiagen	10	1×
crenamoA23F Primer (10 pmol μL^{-1})	1.2	0.6 µM
crenamoA616R Primer (10 pmol μL^{-1})	1.2	0.6 µM
DNA Template (1 ng μL^{-1})	2	0.2 ng μL^{-1}
dd H_2O to 20 µL	5.6	

2. **Set Up Thermocycling Conditions:** Load the reaction tubes into the Bio-Rad CFX Connect Real-Time PCR System and use the following cycling conditions:

Step	Temperature	Time	Cycle(s)
Initial Denaturation	95 °C	15 min	1 cycle
Denaturation	94 °C	15 sec	35 cycles
Annealing	56 °C	30 sec	
Extension	72 °C	60 sec	
Melting Curve Analysis	95 °C	1 min	1 cycle
	65–95 °C	5 sec/0.5°C increment	

3. **Melting Curve Analysis:** After amplification, perform a melting curve analysis from 65 °C to 95 °C with 0.5 °C increments every 5 seconds to confirm the specificity.

2.3.3.3. Quantitative PCR (qPCR) for Comammox amoA gene

This qPCR protocol quantifies the *amoA* gene of Comammox bacteria, and helps assess the abundance of Comammox populations, providing insights into ammonia oxidation dynamics. The qPCR is performed using the primers *comamoAF* and *comamoAR*, following the methods described by Zhao et al. (2019) with slight modifications.

A. Materials and Equipment

A1. Primers:

- *comamoAF* Primer: 5'-AGGNGAYTGGGAYTTCTGG-3'
- *comamoAR* Primer: 5'-CGGACAWABRTGAABCCCAT-3' [W = A or T, Y = C or T, R = A or G]

A2. Reagents:

- KAPA SYBR FAST qPCR Master Mix (2×), Universal
- Bovine Serum Albumin (BSA, 20 mg mL^{-1})
- Template DNA
- Double distilled water (dd H_2O)

A3. Equipment:

- Bio-Rad CFX Connect Real-Time PCR System
- Pipettes and filter tips
- Microcentrifuge tubes

B. Procedure

1. **Prepare the qPCR Reaction Mix:** In a 20 µL reaction volume, combine the following reagents:

Reagent	Volume (µL)	Final Concentration
KAPA SYBR FAST qPCR Master Mix (2×)	10	1×
comamoAF Primer (20 pmol $µL^{-1}$)	0.2	0.2 µM
comamoAR Primer (20 pmol $µL^{-1}$)	0.2	0.2 µM
BSA (20 mg mL^{-1})	0.4	0.4 mg mL^{-1}
DNA Template (10 ng $µL^{-1}$)	2	1 ng $µL^{-1}$
dd H_2O to 20 µL	7.2	

2. **Set Up Thermocycling Conditions:** Load the reaction tubes into the Real-Time PCR System and use the following cycling conditions:

Step	Temperature	Time	Cycle(s)
Initial Denaturation	95 °C	3 min	1 cycle
Denaturation	95 °C	3 sec	40 cycles
Annealing	55 °C	30 sec	
Extension	72 °C	10 sec	
Melting Curve Analysis	95 °C	1 min	1 cycle
	65–95 °C	5 sec/0.5°C increment	

3. **Melting Curve Analysis:** After amplification, perform a melting curve analysis from 65 °C to 95 °C with 0.5 °C increments every 5 seconds to confirm the specificity of the qPCR products.

2.3.3.4. Quantitative PCR (qPCR) for Comammox amoA gene in clades A and B

The following qPCR protocols target the *amoA* gene within Comammox clade A and clade B, utilizing a mix of specific primer sequences to detect and quantify *amoA* gene copies. This quantification assists in determining the abundance and distribution of Comammox clade A and clade B in environmental samples. Reactions are carried out using a Bio-Rad CFX Connect Real-Time PCR System with primers and conditions optimized as per Pjevac et al. (2017).

A. Materials and Equipment

A1. Primers for Comammox Clade A and B:
- Comammox Clade A Primers: *comaA244f* equimolar mix (a-f) and *comaA659r* equimolar mix (a-f)
- Comammox Clade B Primers: *comaB244f* equimolar mix (a-f) and *comaB659r* equimolar mix (a-f)

A2. Reagents:
- KAPA SYBR FAST qPCR Master Mix (2×), Universal
- Bovine Serum Albumin (BSA, 20 mg mL^{-1})
- Template DNA
- Double distilled water (dd H_2O)

A3. Equipment:
- Bio-Rad CFX Connect Real-Time PCR System (or other Real-Time PCR System)
- Pipettes and filter tips
- Microcentrifuge tubes

B. Procedure for Clade A

1. **Prepare the qPCR Reaction Mix for Clade A:**

In a 20 µL reaction volume, combine the following reagents:

Reagent	Volume (µL)	Final Concentration
KAPA SYBR FAST qPCR Master Mix (2×)	10	1×
comaA244f Primer Equimolar Mix, Clade A (16.67 pmol mL^{-1})	0.2	0.17 µM
comaA659r Primer Equimolar Mix, Clade A (16.67 pmol mL^{-1})	0.2	0.17 µM
BSA (20 mg mL^{-1})	0.4	0.4 mg mL^{-1}
DNA Template (10 ng μL^{-1})	2	1 ng μL^{-1}
dd H_2O to 20 µL	7.2	

2. **Set Up Thermocycling Conditions for Clade A:**
Load the reaction tubes into the Bio-Rad CFX Connect Real-Time PCR System and use the following cycling conditions:

Step	Temperature	Time	Cycle(s)
Initial Denaturation	95 °C	3 min	1 cycle
Denaturation	95 °C	3 sec	40 cycles
Annealing	52 °C	20 sec	
Extension	72 °C	1 sec	
Additional Step	80 °C	30 sec	
Melting Curve Analysis	95 °C	1 min	1 cycle
	65–95 °C	5 sec/0.5 °C increment	

C. Procedure for Clade B

1. **Prepare the qPCR Reaction Mix for Clade B:**
In a 20 µL reaction volume, combine the following reagents:

Reagent	Volume (µL)	Final Concentration
KAPA SYBR FAST qPCR Master Mix (2×)	10	1×
comaB244f Primer Equimolar Mix, Clade B (16.67 pmol mL^{-1})	0.2	0.17 µM
comaB659r Primer Equimolar Mix, Clade B (16.67 pmol mL^{-1})	0.2	0.17 µM
BSA (20 mg mL^{-1})	0.4	0.4 mg mL^{-1}
DNA Template (10 ng μL^{-1})	2	1 ng μL^{-1}
dd H_2O to 20 µL	7.2	

2. **Set Up Thermocycling Conditions for Clade B:**
Use the same thermocycling conditions as in Clade A (detailed above) to amplify Comammox Clade B.

2.3.3.5. Quantitative PCR (qPCR) for Nitrobacter nxrB gene

This qPCR protocol quantifies the *nxrB* gene (380 bp) of *Nitrobacter*, a gene encoding the B subunit of enzyme nitrite oxidoreductase involved in nitrite oxidation within the nitrogen cycle. Quantifying *nxrB* in soil samples helps assess the abundance of *Nitrobacter* populations, providing insights into nitrogen processing dynamics. The qPCR is performed using the primers *nxrB1F* and *nxrB1R*, following the methods described by Vanparys et al. (2007).

A. Materials and Equipment

A1. Primers:

- *nxrB1F* Primer: 5'-ACGTGGAGACCAAGCCGGG-3'
- *nxrB1R* Primer : 5'-CCGTGCTGTTGAYCTCGTTGA-3' [Y = C or T]

A2. Reagents:

- KAPA SYBR FAST qPCR Master Mix (2×), Universal
- Bovine Serum Albumin (BSA, 20 mg mL^{-1})
- Template DNA
- Double distilled water (dd H_2O)

A3. Equipment:

- Bio-Rad CFX Connect Real-Time PCR System (or other Real-Time PCR System)
- Pipettes and filter tips
- Microcentrifuge tubes

C. Procedure

1. **Prepare the qPCR Reaction Mix:**

In a 10 µL reaction volume, combine the following reagents:

Reagent	Volume (µL)	Final Concentration
KAPA SYBR FAST qPCR Master Mix (2×)	5	1×
nxrB1F Primer (20 pmol $µL^{-1}$)	0.1	0.2 µM
nxrB1R Primer (20 pmol $µL^{-1}$)	0.1	0.2 µM
BSA (20 mg mL^{-1})	0.2	0.4 mg mL^{-1}
DNA Template (1 ng $µL^{-1}$)	2	0.2 ng $µL^{-1}$
dd H_2O to 10 µL	2.6	

2. **Set Up Thermocycling Conditions:**

Load the reaction tubes into the Bio-Rad CFX Connect Real-Time PCR System and use the following cycling conditions:

Step	Temperature	Time	Cycle(s)
Initial Denaturation	95 °C	3 min	1 cycle

Step	Temperature	Time	Cycle(s)
Denaturation	95 °C	5 sec	40 cycles
Annealing	57 °C	20 sec	
Extension	72 °C	30 sec	
Melting Curve Analysis	95 °C	1 min	1 cycle
	65–95 °C	5 sec/0.5°C increment	

3. **Melting Curve Analysis**
After amplification, perform a melting curve analysis from 65 °C to 95 °C with 0.5 °C increments every 5 seconds to confirm the specificity of the qPCR products.

2.3.3.6. Quantitative PCR (qPCR) for Nitrospira nxrB gene

This qPCR protocol quantifies the *nxrB* gene of *Nitrospira*. Quantifying the *Nitrospira nxrB* gene in soil samples provides insights into the population and activity of *Nitrospira* within the nitrogen cycle. This qPCR protocol is optimized for the Bio-Rad CFX Connect Real-Time PCR System and uses the primers *nxrB169f* and *nxrB638r* as described in Pester et al. (2014).

A. Materials and Equipment

A1. Primers:

- *nxrB169f* Primer: 5′-TAC ATG TGG TGG AAC A-3′
- *nxrB638r* Primer: 5′-CGG TTC TGG TCR ATC A-3′ [R = A or G]

A2. Reagents:

- KAPA SYBR FAST qPCR Master Mix (2×), Universal
- Bovine Serum Albumin (BSA, 20 mg mL^{-1})
- Template DNA
- Double distilled water (dd H_2O)

A3. Equipment:

- Bio-Rad CFX Connect Real-Time PCR System (or other Real-Time PCR System)
- Pipettes and filter tips
- Microcentrifuge tubes

B. Procedure

1. **Prepare the qPCR Reaction Mix:**
In a 10 µL reaction volume, combine the following reagents:

Reagent	Volume (µL)	Final Concentration
KAPA SYBR FAST qPCR Master Mix (2×)	5	1×

Reagent	Volume (µL)	Final Concentration
nxrB169f Primer (20 pmol µL^{-1})	0.1	0.2 µM
nxrB638r Primer (20 pmol µL^{-1})	0.1	0.2 µM
BSA (20 mg mL^{-1})	0.2	0.4 mg mL^{-1}
DNA Template (1 ng µL^{-1})	2	0.2 ng µL^{-1}
dd H_2O to 10 µL	2.6	

2. **Set Up Thermocycling Conditions:**
Load the reaction tubes into the Bio-Rad CFX Connect Real-Time PCR System and use the following cycling conditions

Step	Temperature	Time	Cycle(s)
Initial Denaturation	95 °C	3 min	1 cycle
Denaturation	95 °C	5 sec	40 cycles
Annealing	56.2 °C	30 sec	
Extension	72 °C	30 sec	
Melting Curve Analysis	95 °C	1 min	1 cycle
	65–95 °C	sec/0.5°C increment	

3. **Melting Curve Analysis**
After amplification, perform a melting curve analysis from 65 °C to 95 °C with 0.5 °C**.**

D. General Expected Results

The expected outcome is a quantitative assessment of the target gene (*amoA* for AOA, AOB, and comammox bacteria; *nxrB* for *Nitrobacter* and *Nitrospira* nitrite-oxidizing bacteria), indicated by fluorescence data and Ct (cycle threshold) values that represent gene abundance. Melting curve analysis should produce a single peak, confirming specific amplification of the target gene.

D. Troubleshooting and Notes

1. **Inconsistent Melting Curve**: Additional peaks in the melting curve may indicate non-specific amplification. To enhance specificity, increase the annealing temperature or adjust primer concentrations as needed. When using equimolar mixtures of Comammox clade A and B *amoA* primer sets, it may be necessary to test individual primers per soil sample to identify and eliminate combinations that lead to non-specific amplification.

2. **Low Amplification Efficiency**: Check that all reagents are fresh, and primer concentrations are accurate. The addition of BSA helps stabilizing reactions. Adjusting BSA concentration may further improve amplification efficiency.

3. **Template DNA Quality**: High-purity DNA is essential for reliable amplification. Low-quality or impure DNA can inhibit PCR, affecting Ct values and overall results. Using DNA of high purity and free from contaminants is recommended for optimal performance.

2.4. Soil Microbial Diversity Assessment

This module outlines protocols to assess the diversity of soil microbial communities, focusing on nitrifying microorganisms such as AOA, AOB, and comammox bacteria. High-throughput sequencing of the *amoA* gene enables profiling of microbial taxa involved in nitrogen cycling, allowing detailed exploration of nitrifier diversity in soil samples. The provided protocols amplify *amoA* segments (but can be generalized to other markers as ell) for a multiplex Next-Generation Sequencing (NGS) approach to capture variations within and among these nitrifying groups, providing high-resolution community profiling. Two main protocols are provided, an Illumina platform ready-to-go streamlined protocol, and a hyper-multiplex approach (originally developed for low budget laboratories and based on the 454 proposed pre-indexing) which is platform independent and requires further instrument specific adapter ligation.

2.4.1. Illlumina streamlined Protocol for Library Preparation of AOB, AOA, and Comammox

A. Materials and Equipment

A1. Primers:

- **AOB**
  - ILL_amoA1F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGGGTTTCTACTGGTGGT-3'
  - ILL_amoA2R: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCCCTCKGSAAAGCCTTCTTC-3'

- **AOA**
  - I ILL_crenamoA23F: 5' -TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATGGTCTGGCTWAGACG- 3'
  - ILL_crenamoA616R: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCCATCCATCTGTATGTCCA-3'

- **Comammox**
  - ILL_comamoAF: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGNGAYTGGGAYTTCTGG-3'
  - *ILL_comamoAR:* 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGGACAWABRTGAABCCCAT-3'

A2. Reagents:

- MiSeq Reagent Kit v2, 500-cycles
- Platinum Taq DNA Polymerase
- Nextera XT Index Kit v2
- AMPure XP beads
- Freshly Prepared 80% Ethanol
- 10 mM TRIS buffer pH 8.5
- Bioanalyzer 1000 Chip and reagents

A3. Equipment:

- PCR System
- Pipettes and filter tips
- Microcentrifuge tubes

- Magnetic rack
- Shaker
- µDrop
- Plate reader

B. Procedure

B1.1. Step 1: PCR Amplification of *amoA* gene (AOB)

1. **Prepare Reaction Mix for the First PCR (25 µL total volume):**

Reagent	Volume (µL)	Final concentration
10x Platinum Taq DNA Polymerase Buffer	2.5	1x
Forward Primer (ILL_amoA1F, 10 µM)	3	1.2 µM
Reverse Primer (ILL_amoA2R, 10 µM)	3	1.2 µM
dNTPs (10 mM)	0.5	0.2 mM
$MgCl_2$ (50mM)	0.75	1.5 mM
BSA (3%)	0.75	2%
DNA Template (5 ng μL^{-1})	2	10 ng μL^{-1}
Platinum Taq DNA Polymerase (10 U)	0.1	1 U
dd H_2O to 20 µL	12.4	

2. **Thermocycling Conditions for the First PCR:**

Step	Temperature	Time	Cycle(s)
Initial Denaturation	95 °C	15 min	1
Denaturation	95 °C	15 sec	35
Annealing	60 °C	30 sec	
Extension	72 °C	30 sec	
Final Extension	72 °C	10 min	1
Hold	4 °C	∞	

B1.2. Step 1: PCR Amplification of *amoA* gene (AOA)

1. **Prepare Reaction Mix for the First PCR (25 µL total volume):**

Reagent	Volume (µL)	Final concentration
10x Platinum Taq DNA Polymerase Buffer	2.5	1x
Forward Primer (ILL_amoA1F, 10 µM)	1.5	0.6 µM
Reverse Primer (ILL_amoA2R, 10 µM)	1.5	0.6 µM

Reagent	Volume (µL)	Final concentration
dNTPs (10 mM)	0.5	0.2 mM
$MgCl_2$ (50mM)	0.75	1.5 mM
BSA (3%)	0.75	2%
DNA Template (5 ng μL^{-1})	2	10 ng μL^{-1}
Platinum Taq DNA Polymerase (10 u)	0.1	1 u
dd H_2O to 20 µL	15.4	

2. Thermocycling Conditions for the First PCR:

Step	Temperature	Time	Cycle(s)
Initial Denaturation	95 °C	15 min	1
Denaturation	95 °C	15 sec	35
Annealing	56 °C	30 sec	
Extension	72 °C	30 sec	
Final Extension	72 °C	10 min	1
Hold	4 °C	∞	

B1.3. Step 1: PCR Amplification of *amoA* gene (Comammox)

1. Prepare Reaction Mix for the First PCR (25 µL total volume):

Reagent	Volume (µL)	Final concentration
10x Platinum Taq DNA Polymerase Buffer	2.5	1x
Forward Primer (ILL_amoA1F, 10 µM)	2.5	1 µM
Reverse Primer (ILL_amoA2R, 10 µM)	2.5	1 µM
dNTPs (10 mM)	0.5	0.2 mM
$MgCl_2$ (50mM)	0.75	1.5 mM
BSA (3%)	0.75	2%
DNA Template (5 ng μL^{-1})	2	10 ng μL^{-1}
Platinum Taq DNA Polymerase (10 u)	0.1	1 u
dd H_2O to 20 µL	13.4	

2. Thermocycling Conditions for the First PCR:

Step	Temperature	Time	Cycle(s)
Initial Denaturation	95 °C	15 min	1
Denaturation	95 °C	15 sec	40
Annealing	61 °C	30 sec	
Extension	72 °C	30 sec	
Final Extension	72 °C	10 min	1
Hold	4 °C	∞	

B2. PCR Clean up

The PCR products from the initial amplification are purified using AMPure XP beads to remove residual primers and primer dimers.

B2.1. Procedure

1. Incubate AMPure XP beads at room temperature for 30 minutes.
2. Centrifuge the amplicon PCR product at 1,000 × g for 1 minute at 20°C to collect condensation.
3. Vortex the AMPure XP beads for 30 seconds to ensure even dispersion.
4. Add 20 µL of AMPure XP beads to each sample.
5. Gently pipette the entire volume up and down 10 times.
6. Incubate the samples at room temperature without shaking for 5 minutes.
7. Place the samples on a magnetic stand until the supernatant is clear.
8. While on the magnetic stand, carefully remove and discard the supernatant.
9. Wash the beads with freshly prepared 80% ethanol as follows:
    a. Add 200 µL of freshly prepared 80% ethanol to each sample.
    b. Incubate on the magnetic stand for 30 seconds.
    c. Carefully remove and discard the supernatant.
10. Repeat the ethanol wash step.
11. With the samples on the magnetic stand, allow the beads to air-dry for 10 minutes.
12. Remove the samples from the magnetic stand and add 27.5 µL of 10 mM Tris, pH 8.5, to each sample.
13. Gently pipette up and down 10 times to ensure the beads are fully resuspended.
14. Incubate at room temperature for 2 minutes.
15. Place the samples back on the magnetic stand until the supernatant is clear.
16. Carefully transfer 25 µL of the supernatant from each cleaned sample to a new tube.

B3. Step 2: Indexing PCR for *amoA* gene (AOB, AOA, Comammox)

- **ILLUMINA NEXTERA v2 Index sets and plate set up**
  Set A: N701-715, S502-511
  Set B: N716-729, S502-511
  Set C: N701-715, S513-522
  Set D: N716-729, S513-522

TABLE II. Plate set up of the different index primers sets.

Index Set A												
	N701	**N702**	**N703**	**N704**	**N705**	**N706**	**N707**	**N710**	**N711**	**N712**	**N714**	**N715**
S502												
S503												
S505												
S506												
S507												
S508												
S510												
S511												

Index Set B												
	N716	**N718**	**N719**	**N720**	**N721**	**N722**	**N723**	**N724**	**N726**	**N727**	**N728**	**N729**
S502												
S503												
S505												
S506												
S507												
S508												
S510												
S511												

Index Set C												
	N701	**N702**	**N703**	**N704**	**N705**	**N706**	**N707**	**N710**	**N711**	**N712**	**N714**	**N715**
S513												
S515												
S516												
S517												
S518												
S520												
S521												
S522												

	Index Set D											
	N716	**N718**	**N719**	**N720**	**N721**	**N722**	**N723**	**N724**	**N726**	**N727**	**N728**	**N729**
S513												
S515												
S516												
S517												
S518												
S520												
S521												
S522												

TABLE III. Primer NEXTERA v2 index sequences for the Read 1.

Name	Nextera adapter	Unique identifier (i7)	P7 sequence
N701	CAAGCAGAAGACGGCATACGAGAT	TCGCCTTA	GTCTCGTGGGCTCGG
N702		CTAGTACG	
N703		TTCTGCCT	
N704		GCTCAGGA	
N705		AGGAGTCC	
N706		CATGCCTA	
N707		GTAGAGAG	
N710		CAGCCTCG	
N711		TGCCTCTT	
N712		TCCTCTAC	
N714		TCATGAGC	
N715		CCTGAGAT	
N716		TAGCGAGT	
N718		GTAGCTCC	
N719		TACTACGC	
N720		AGGCTCCG	
N721		GCAGCGTA	
N722		CTGCGCAT	
N723		GAGCGCTA	
N724		CGCTCAGT	
N726		GTCTTAGG	
N727		ACTGATCG	
N728		TAGCTGCA	
N729		GACGTCGA	

TABLE IIII. Primer NEXTERA v2 index sequences for the Read 2.

Name	Nextera adapter	Unique identifier (i5)	P5 sequence
S502	AATGATACGGCGACCACCGAGATCTACAC	TCGCCTTA	TCGTCGGCAGCGTC
S503		CTAGTACG	
S505		TTCTGCCT	
S506		GCTCAGGA	
S507		AGGAGTCC	
S508		CATGCCTA	
S510		GTAGAGAG	
S511		CAGCCTCG	
S513		TGCCTCTT	
S515		TCCTCTAC	
S516		TCATGAGC	
S517		CCTGAGAT	
S518		TAGCGAGT	
S520		GTAGCTCC	
S521		TACTACGC	
S522		AGGCTCCG	

1. **Prepare Reaction Mix for the Second PCR (25 µL total volume):**

Reagent	Volume (µL)	Final concentration
10x Platinum Taq DNA Polymerase Buffer	2.5	1x
Index (N7XX)	2.5	1 µM
Index (S5XX)	2.5	1 µM
dNTPs (10 mM)	0.5	0.2 mM
$MgCl_2$ (50mM)	0.75	1.5 mM
DNA Template	5	
Platinum Taq DNA Polymerase (10 u)	0.1	1 u
dd H_2O to 20 µL	11.15	

2.Thermocycling Conditions for the Second PCR:

Step	Temperature	Time	Cycle(s)
Initial Denaturation	95 °C	3 min	1
Denaturation	95 °C	30 sec	8
Annealing	60 °C	55 sec	
Extension	72 °C	72 sec	
Final Extension	72 °C	5 min	1
Hold	4 °C	∞	

B4. PCR Clean up

The PCR products from the indexing amplification are purified using AMPure XP beads, as described in Section B2.

B5. Equimolar pooling

The absorbance of the samples at 260 nm is measured using a µDrop plate and plate reader. Normalization of these measurements is required to calculate the volume (µL) needed from each sample to create an equimolar pool of all samples

Procedure:

1. Transfer 2 µL of the samples to the µDrop.
2. Measure the absorbance at 260 nm.
3. To create an equimolar pool of your samples, set the volume of the most concentrated sample (Sample X) to 1 µL, then calculate the transfer volumes for the remaining samples based on their concentration ratios to Sample X
4. Transfer calculated volumes of each sample within a defined pool in a new 1.5 mL DNA low bind reaction tube.

2.4.2. Platform independent protocol for preparing hyper-multiplexed libraries

This protocol is based on the simple addition of a 5' overhang sample specific index to one or both amplification primers, allowing hyper multiplexing of samples and primers per final library, with both the index and primers facilitating the post-sequencing demultiplexing process. Library prep is completed via the addition of the platform specific adapters with the help of associated kits (e.g. for Illumina, the KAPA HyperPrep ligation kit at a PCR-free mode, or other equivalents for other platforms – e.g. PacBio or Nanopore), a process usually performed by sequencing vendors unless an instrument for the method is available. Its design was based on the older "barcoded pyrosequencing" approach (Hamady and Knight, 2009). It relies on a 2-setp indexing process, where a 28-cycle PCR is employed for amplifying each sample with the index free primers (to avoid possible bias induced by primer indexes), followed by a 7-cycle

sample-specific labelling PCR with the indexed primers. It has been routinely employed in our group for targeting an array of marker genes depending on the primer sets used (e.g. Vasileiadis et al., 2018). Demultiplexing post-sequencing of the retrieved reads to their samples of origin and retrieval of the analysis and repository (e.g. NCBI) submission-ready is performed with the help of a Bash/sh script found at the Github annex of our previous work (Vasileiadis et al., 2022). The script named "DemuxOwnBCsys_absPATH.sh", accounts for the possibility of the index being read by either the forward or the reverse read, it relies on the Flexbar software (Dodt et al., 2012), and can be found at e.g. https://github.com/SotiriosVasileiadis/mconsort_tbz_degr/tree/master/4_microbiomes/1_fetch_and_demultiplex/1_tbz_vs_suc/2_demultiplexed_sequences (instructions are provided after running the script without any arguments). Following, the process employed for the *amoA* genes of AOA, AOB, and Comammox bacteria is provided.

2.4.2.1. Protocol for Ammonia-Oxidizing Bacteria (AOB)

A. Materials and Equipment

A1. Primers:

- amoA-1F Primer: 5'-GGGGTTTCTACTGGTGGT-3'
- amoA-2R Primer: 5'-CCCCTCKGSAAAGCCTTCTTC-3' [K = G or T; S = G or C]
- Forward Index Primer: 5'-NNNNNNNAAGGGGTTTCTACTGGTGGT-3' (See Table I.)

A2. Reagents:

- Q5® High-Fidelity 2X Master Mix (New England Biolabs)
- Bovine Serum Albumin (BSA, 20 mg mL^{-1})
- Template DNA
- Double distilled water (dd H_2O)

A3. Equipment:

- PCR System
- Pipettes and filter tips
- Microcentrifuge tubes

B. Procedure

B1. Step 1: PCR Amplification of *amoA* gene (AOB)

1. **Prepare Reaction Mix for the First PCR (20 µL total volume):**

Reagent	Volume (µL)	Final concentration
PCR mix (2×)	10	1x
Forward Primer (*amoA-1F*, 10 µM)	1	0.5 µM
Reverse Primer (amoA-2R, 10 µM)	1	0.5 µM
BSA (20 mg mL^{-1})	0.4	0.4 mg mL^{-1}

Reagent	Volume (µL)	Final concentration
DNA Template (1 ng μL^{-1})	2	0.1 ng μL^{-1}
dd H_2O to 20 µL	5.6	

2. **Thermocycling Conditions for the First PCR:**

Step	Temperature	Time	Cycle(s)
Initial Denaturation	98 °C	30 sec	1
Denaturation	98 °C	10 sec	30
Annealing	54 °C	30 sec	
Extension	72 °C	1 min	
Final Extension	72 °C	10 min	1
Hold	4 °C	∞	

B2. Step 2: Indexing PCR for *amoA* gene (AOB)

1. **Prepare Reaction Mix for the Second PCR (20 µL total volume):**

Reagent	Volume (µL)	Final Concentration
PCR mix (2×)	10	1x
Forward Index Primer (10 µM)	1	0.5 µM
Reverse Primer (amoA-2R, 10 µM)	1	0.5 µM
DNA Template from 1st PCR	2	
dd H_2O to 20 µL	6	

2. **Thermocycling Conditions for the Second PCR:**

Step	Temperature	Cycle(s)
Initial Denaturation	98 °C	1
Denaturation	98 °C	7
Annealing	54 °C	
Extension	72 °C	
Final Extension	72 °C	1
Hold	4 °C	

2.4.2.2. Protocol for Ammonia-Oxidizing Archaea (AOA)

A. Materials and Equipment

A1. Primers:

- amoA-310f: 5'- GGTGGATACCBTCWGCAATG-3'
- amoA-529r: 5'-GCAACMGGACTATTGTAGAA-3' (Marushenko et al., 2013)
- Forward Index Primer: 5'-NNNNNNNNNGGTGGATACCBTCWGCAATG-3' (See Table I.)

A2. Reagents:

- Q5® High-Fidelity 2X Master Mix (New England Biolabs)
- Bovine Serum Albumin (BSA, 20 mg mL^{-1})
- Template DNA
- Double distilled water (dd H_2O)

A3. Equipment:

- PCR System
- Pipettes and filter tips
- Microcentrifuge tubes

B. Procedure

B1. Step 1: PCR Amplification of *amoA* gene (AOA)

1. **Prepare Reaction Mix for the First PCR (20 µL total volume):**

Reagent	Volume (µL)	Final concentration
PCR mix (2×)	10	1x
Forward Primer (amoA-310f, 10 µM)	1	0.5µM
Reverse Primer (amoA-529r, 10 µM)	1	0.5µM
BSA (20 mg mL^{-1})	0.4	0.4 mg mL^{-1}
DNA Template (1 ng μL^{-1})	2	0.1 ng μL^{-1}
dd H_2O to 20 µL	5.6	

2. **Thermocycling Conditions for the First PCR:**

Step	Temperature	Cycle(s)
Initial Denaturation	98 °C	1
Denaturation	98 °C	30

Step	Temperature	Cycle(s)
Annealing	54 °C	
Extension	72 °C	
Final Extension	72 °C	1
Hold	4 °C	

B2. Step 2: Indexing PCR for *amoA* gene (AOA)

1. **Prepare Reaction Mix for the Second PCR (20 µL total volume):**

Reagent	Volume (µL)	Final concentration
PCR mix (2×)	10	1x
Forward Index Primer (10 µM)	1	0.5 µM
Reverse Primer (amoA-529r, 10 µM)	1	0.5 µM
DNA Template from 1st PCR	2	
dd H_2O to 20 µL	6	

2. **Thermocycling Conditions for the Second PCR:**

Step	Temperature	Cycle(s)
Initial Denaturation	98 °C	1
Denaturation	98 °C	7
Annealing	54 °C	
Extension	72 °C	
Final Extension	72 °C	1
Hold	4 °C	

2.4.2.3. Protocol for Comammox amoA gene Amplification

A. Materials and Equipment

A1. Primers:

- comamoAF Primer: 5'-AGGNGAYTGGGAYTTCTGG-3'
- comamoAR Primer: 5'-CGGACAWABRTGAABCCCAT-3' [W = A or T, Y = C or T, R = A or G]
- Forward Index Primer: 5'-NNNNNNNNNAGGNGAYTGGGAYTTCTGG-3' (See Table I.)

A2. Reagents:

- Q5® High-Fidelity 2X Master Mix (New England Biolabs)
- Bovine Serum Albumin (BSA, 20 mg mL^{-1})
- Template DNA

- Double distilled water (dd H_2O)

A3. Equipment:

- PCR System
- Pipettes and filter tips
- Microcentrifuge tubes

B. Procedure

B1. Step 1: PCR Amplification of Comammox *amoA* gene

1. Prepare Reaction Mix for the First PCR (20 µL total volume):

Reagent	Volume (µL)	Final concentration
PCR mix (2×)	10	1x
Forward Primer (*comamoAF*, 10 µM)	1	0.5 µM
Reverse Primer (*comamoAR*, 10 µM)	1	0.5 µM
Bovine Serum Albumin (BSA, 20 mg mL^{-1})	0.4	0.4 mg mL^{-1}
DNA Template (10-25 ng μL^{-1})	2	1-2.5 ng μL^{-1}
dd H_2O to 20 µL	5.6	

2. Thermocycling Conditions for the First PCR:

Step	Temperature	Cycles
Initial Denaturation	98 °C	1
Denaturation	98 °C	30
Annealing	50 °C	
Extension	72 °C	
Final Extension	72 °C	1
Hold	4 °C	

B2. Step 2: Indexing PCR for Comammox *amoA* gene

1. Prepare Reaction Mix for the Second PCR (20 µL total volume):

Reagent	Volume (µL)	Final concentration
PCR mix (2×)	10	1x
Forward Index Primer (10 µM)	1	0.5 µM
Reverse Primer (*comamoAR*, 10 µM)	1	0.5 µM
DNA Template from First PCR	2	
dd H_2O to 20 µL	6	

2. Thermocycling Conditions for the Second PCR:

Step	Temperature	Cycle(s)
Initial Denaturation	98 °C	1
Denaturation	98 °C	7
Annealing	50 °C	
Extension	72 °C	
Final Extension	72 °C	1
Hold	4 °C	

TABLE I. Primer index sequences (5′ - 3′) (represented by Ns) associated with the primers (i) amoA-1f for AOB; (ii) amoA-310f for AOA, and (iii) comamoA-F for Comammox.

No. primer index	AOB amoA-1f	AOA amoA-310f	Comammox comamoA- F
1	TTATTACGC	TTATTACCG	TTATTACGG
2	TTATTCGTC	TTATTAGGC	TTATTCTCG
3	TTATATGGC	TTATACTCC	TTATTGCCT
4	TTATCTCTC	TTATACAGG	TTATTGGTG

5	TTATCCTAC	TTATAGCTG	TTATAAGCG
6	TTATCGAGC	TTATAGGAC	TTATACCTG
7	TTATGGTTC	TTATGTTGG	TTATAGTGG
8	TTAATCTGC	TTATGTCTC	TTATCTAGG
9	TTAATGCTC	TTATGAACC	TTATCTGCT
10	TTAAGCATC	TTATGACGT	TTATCGTCA
11	TTACTTGAC	TTATGAGAG	TTAATTGCG
12	TTACAATGC	TTATGGTCA	TTAATCGGT
13	TTACGTCGC	TTAATCTCG	TTAACTCTG
14	TTCTATCCT	TTAATCAGC	TTAACATCG
15	TTCTACTTC	TTAATGGTG	TTAACGTGT
16	TTCTACGAT	TTAACTACC	TTAACGCAA
17	TTCTCTTGC	TTAACTCTG	TTAAGTTGG
18	TTCTCTGTT	TTAACTGGA	TTAAGCTCA
19	TTCTCATCT	TTAACAAGG	TTAAGCCTT
20	TTCTCGCAT	TTAACACAC	TTAAGGACT
21	TTCTGTGAC	TTAACCGTT	TTACTTCGT
22	TTCTGACTC	TTAACGTAG	TTACTAACG
23	TTCTGGATT	TTAACGCCT	TTACTCGCA
24	TTCATTCGC	TTAAGTCCA	TTACTGGAT
25	TTCATCGTT	TTAAGTGAC	TTACAACCT
26	TTCATGTCT	TTAAGAGCT	TTACACTGT
27	TTCATGGAC	TTAAGCATG	TTACCTACA
28	TTCAAGCTT	TTAAGGAGT	TTACGTTCT
29	TTCCGTTAT	TTACTTACG	TTACGATTG
30	TTCCGAGCT	TTACTTGTC	TTACGACAA
31	TTCCGCTGC	TTACTATGG	TTACGAGGT
32	TTCCGCCTT	TTACTAGCA	TTACGGCGG
33	TTCGTTACT	TTACTCCTT	TTAGTAGCT

34	TTCGTTGTC	TTACTCGAG	TTAGTGACA
35	TTCGTCTAT	TTACATCAC	TTAGCAAGT
36	TTCGTGTGC	TTACAACCT	TTAGCCTAT
37	TTCGACATT	TTACAAGTG	TTAGCGTTG
38	TTCGACGGC	TTACACTGT	TTAGGTACG
39	TTCGCATAC	TTACACATC	TTAGGTCAT
40	TTCGCCGCT	TTACAGTCG	TTAGGCGTG
41	TTCGCGATC	TTACAGAGA	TTCTTAGCA
42	TTGTTCTCT	TTACCTAGT	TTCTTCTGT
43	TTGTTGCTT	TTACCACTA	TTCTTGACG
44	TTGTCTACT	TTACCAGAT	TTCTTGCTA
45	TTGTGTCAT	TTACCGAAC	TTCTATTCG
46	TTGTGCGTT	TTACCGCGG	TTCTAAGGT
47	TTGATTCCT	TTACGATAC	TTCTACACT
48	TTGACTGAT	TTACGCTTA	TTCTACGTA
49	TTGAGCTAT	TTACGCAAT	TTCTCTCTT
50	TTGAGGACT	TTACGCCGC	TTCTCATTG
51	TTGCTTGTT	TTAGTACGA	TTCTCACAA
52	TTGCTACAT	TTAGTCTAC	TTCTCGAGT
53	TTGCATTCT	TTAGTCGCT	TTCTGTACA
54	TTGCCGATT	TTAGTGTGT	TTCTGTCGG
55	TTGGTGGAT	TTAGTGATC	TTCTGTGAT
56	TTGGATCTT	TTAGTGGAA	TTCTGATCT
57	TTGGCGTCT	TTAGATAGC	TTCTGCGCG
58	TATTAAGGC	TTAGAACTC	TTCTGGTAA
59	TATTCTCCT	TTAGAAGGT	TTCATTAGG
60	TATTCTGAC	TTAGACTTG	TTCATACTG
61	TATTCCTTC	TTAGACCAT	TTCAATCGT
62	TATTCGCGC	TTAGAGAAG	TTCAACTTG

63	TATTCGGTT	TTAGCTCAA	TTCAACGAT
64,	TATTGTTGC	TTAGCATCT	TTCAAGTCT
65	TATTGAGCT	TTAGCCTGA	TTCACTTCA
66	TATTGGATC	TTAGCCACG	TTCACAACT
67	TATATCCTC	TTAGCGGCC	TTCACGCGG
68	TATAATCGC	TTAGGCGTC	TTCACGGTT
69	TATAAGCCT	TTGTTAAGG	TTCGTTACT
70	TATAAGGTC	TTGTTACTC	TTCGTTCAA
71	TATACATGC	TTGTTGTTG	TTCGTTGTG
72	TATCTTGTC	TTGTTGAAC	TTCGTATCG
73	TATCTAAGC	TTGTTGGCT	TTCGTCTTA
74	TATCTCGCT	TTGTAATCG	TTCGTGTAT
75	TATCCGTCT	TTGTACTGA	TTCGACCTT
76	TATCGATTC	TTGTACACT	TTCGAGGCA
77	TATCGCGAC	TTGTACCAG	TTCGCTTGG
78	TATCGGCTT	TTGTACGTC	TTCGCTATA
79	TATGTCTGC	TTGTAGCGC	TTCGCGCCT
80	TATGTGACT	TTGTCTTAC	TTCGGACTA
81	TATGATGCT	TTGTCTATG	TTCGGCAAT
82	TATGACATC	TTGTCTCCT	TTGTTCGTT
83	TATGGACGC	TTGTCAACA	TTGTAGCAT
84	TATGGCTCT	TTGTCAGTT	TTGTCTTGT
85	TAATTGTGC	TTGTCCGAA	TTGTCAACG
86	TAATTGCCT	TTGTCGTGT	TTGTCGATA
87	TAATACGAC	TTGTGTAGT	TTGTGTCTA
88	TAATCCGCT	TTGTGTCAA	TTGTGACGT
89	TAATGTGTC	TTGTGAGGA	TTGTGCTGG
90	TAAGCTTAC	TTGTGGATA	TTGATCTCT
91	TAAGCAAGC	TTGTGGCCG	TTGACTCAT

92	TAAGCCGTC	TTGATAACC	TTGACTGTA
93	TAAGCGCAT	TTGATAGAG	TTGACCTAA
94	TAAGGTCCT	TTGATCTGT	TTGAGTAGT
95	TAAGGCAAT	TTGATCGCA	TTGAGCACG
96	TACTATCTC	TTGAATACG	TTGAGGTTA

2.4.2.4.PCR Clean up and Equimolar Pooling

This protocol describes the process for equimolar pooling of PCR products and subsequent clean-up using SPRI beads to prepare a sequencing-ready pool.

A. Procedure

1. Verify uniformity of PCR products by assessing band intensity on a gel or through another quantification method.
2. Combine a specific volume of each PCR product to create the pool.
3. Adjust the volume of individual products as needed to account for variability in amplification efficiency.
4. Add SPRI beads at a ratio of 1.8× the total volume of the pooled PCR products.
5. Mix by gently pipetting up and down 10 times.
6. Incubate the sample at room temperature for 5 minutes to allow the DNA to bind to the beads.
7. Place the sample on a magnetic stand until the supernatant is clear.
8. Carefully remove and discard the supernatant without disturbing the beads.
9. Add 200 µL of 70% ethanol to the sample while it remains on the magnetic stand.
10. Incubate for 30 seconds, then carefully remove and discard the ethanol.
11. Repeat the ethanol wash step once more.
12. Allow the beads to air-dry on the magnetic stand for 5–10 minutes.
13. Remove the sample from the magnetic stand and add an appropriate buffer or nuclease-free water (e.g., 100 µL).
14. Gently pipette up and down 10 times to resuspend the beads.
15. Incubate at room temperature for 2 minutes.
16. Place the sample back on the magnetic stand until the supernatant is clear.
17. Transfer 50 µL of the supernatant (sequencing-ready pool) to a new tube.
18. Aliquot ~45 µL as a backup if desired.
19. Measure the DNA concentration to confirm sufficient quantity and quality for downstream sequencing applications.

3. Concluding Remarks

The *Modular-based Protocol for Ecogenomics Application in N Cycle* manual in its current state reflects the state-of-the-art methodologies within the ACTIONr project, serving as a structured guide for research on nitrogen cycling. This evolving resource will be continuously updated, incorporating new insights and protocol refinements to maintain its relevance for both current and future studies.

Future editions will expand upon these protocols, including cultivation protocols for synthetic microbial communities of nitrifiers, and detailed measurements for greenhouse gas emissions (CO_2, CH_4, N_2O). Additionally, optimized RNA extraction methods will be provided to advance transcriptomic analyses, with protein extraction protocols to be introduced in future editions once proteomics methods are fully established, enabling comprehensive dual-omics studies of ammonia-oxidizing microorganisms. By standardizing these practices, ACTIONr aims to ensure reproducible and adaptable methods, aligning with its objective to provide robust, standardized procedures for nitrogen cycle research.

4. References

Aigle, A., Prosser, J.I., & Gubry-Rangin, C. (2019). The application of high-throughput sequencing technology to analysis of *amoA* phylogeny and environmental niche specialisation of terrestrial bacterial ammonia-oxidisers. Environ Microbiome, 14, 1-10. https://doi.org/10.1186/s40793-019-0342-6.

Alves, R.J.E., Minh, B.Q., von Haeseler, A., & Schleper, C. (2018). Unifying the global phylogeny and environmental distribution of ammonia-oxidising archaea based on *amoA* genes. Nat Commun, 9, 1517. https://doi.org/10.1038/s41467-018-03861-1.

Brochier-Armanete, C., Boussau, B., Gribaldo, S., & Forterre, P. (2008). Mesophilic Crenarchaeota: Proposal for a Third Archaeal Phylum, the Thaumarchaeota. Nat Rev Microbiol, 6, 245-252. https://doi.org/10.1038/nrmicro1852.

Campbell, M.A., Chain, P.S., Dang, H., El Sheikh, A.F., Norton, J.M., Ward, N.L., & Klotz, M.G. (2011). *Nitrosococcus watsonii* sp. nov., a new species of marine obligate ammonia-oxidizing bacteria that is not omnipresent in the world's oceans: Calls to validate the names '*Nitrosococcus halophilus*' and '*Nitrosomonas mobilis*'. FEMS Microbiol, 76, 39–48. https://doi.org/10.1111/j.1574-6941.2010.01027.x.

Chain, P., Lamerdin, J., Larimer, F., Regala, W., Lao, V., Land, M., Hauser, L., Hooper, A., Klotz, M., Norton, J., Sayavedra-Soto, L., Arciero, D., Hommes, N., Whittaker, M., & Arp, D. (2003). Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. J Bacteriol, 185, 2759-73. https://doi.org/10.1128/JB.185.9.2759-2773.2003.

Doane, T.A., & Horwáth, W.R. (2003). Spectrophotometric determination of nitrate with a single reagent. Anal Lett, 36, 2713–2722. https://doi.org/10.1081/AL-120024647.

Dodt, M, Roehr, J T, Ahmed, R, Dieterich, C (2012). FLEXBAR—Flexible Barcode and Adapter Processing for Next-Generation Sequencing Platforms. Biology 1, 895-905, 10.3390/biology1030895

DSMZ. (n.d.). Available from: https://bacmedia.dsmz.de/medium/1583.

Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., & Oakley, B.B. (2005). Ubiquity and diversity of ammonia-oxidizing archaea in water column sand sediments of the ocean. PNAS USA, 102, 14683–14688. https://doi.org/10.1073/pnas.0506625102.

Gubry-Rangin, C., Nicol, G.W., & Prosser, J.I. (2010). Archaea rather than bacteria control nitrification in two agricultural acidic soils. FEMS Microbiol Ecol, 74, 566-574. https://doi.org/10.1111/j.1574-6941.2010.00971.x.

Hamady, M, Knight, R (2009). Microbial community profiling for human microbiome projects: tools, techniques, and challenges. Genome Res 1910.1101/gr.085464.108

Hayatsu, M., Tago, K., Uchiyama, I., Toyoda, A., Wang, Y., Shimomura, Y., et al. (2017). An acid-tolerant ammonia-oxidizing γ-proteobacterium from soil. ISME J, 11, 1130–1141.

Head, I.M., Hiorns, W.D., Embley, T.M., McCarthy, A.J., Saunders, J.R. (1993). The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J Gen Microbiol, 139, 1147-1153.

Hink, L., Gubry-Rangin, C., Nicol, G.W., & Prosser, J.I. (2018). The consequences of niche and physiological differentiation of archaeal and bacterial ammonia oxidisers for nitrous oxide emissions. ISME J, 12, 1084–1093.

Hodgskiss, L.H., Melcher, M., Kerou, M., et al. (2023). Unexpected complexity of the ammonia monooxygenase in archaea. ISME J, 17, 588–599. https://doi.org/10.1038/s41396-023-01367-3.

Kandeler, E., & Gerber, H. (1988). Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fertil Soil, 6, 68-72.

Kerou, M., Alves, R.J.E., & Schleper, C. (2016). Genus *Nitrososphaera*. In W.B. Whitman (Ed.), Bergey's manual of systematics of archaea and bacteria. Hoboken, NJ: John Wiley & Sons Inc. https://doi.org/10.1002/9781118960608.cbm00055.

Kozlowski, J.A., Kits, K.D., & Stein, L.Y. (2016). Complete genome sequence of *Nitrosomonas ureae* strain Nm10, an oligotrophic group 6a nitrosomonad. Genome Announcements, 4, e00094-16. https://doi.org/10.1128/genomea.00094-16.

Lehtovirta-Morley, L.E., Stoecker, K., Vilcinskas, A., Prosser, J.I., & Nicol, G.W. (2011). Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. PNAS, 108, 15892-15897. https://doi.org/10.1073/pnas.1107196108.

Lehtovirta-Morley, L.E., Verhamme, D.T., Nicol, G.W., & Prosser, J.I. (2014). Effect of nitrification inhibitors on the growth and activity of *Nitrosotalea devanaterra* in culture and soil. Soil Biol Biochem, 62, 129-133. https://doi.org/10.1016/j.soilbio.2013.01.020.

Marusenko, Y., Bates, S.T., Anderson, I., Johnson, S.L., Soule, T., & Garcia-Pichel, F. (2013). Ammonia-oxidizing archaea and bacteria are structured by geography in biological soil crusts across North American arid lands. Ecol Process, 2, 9. https://doi.org/10.1186/2192-1709-2-9.

Mobarry, B.K., Wagner, M., Urbain, V., Rittmann, B.E., & Stahl, D.A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl Environ Microbiol, 62, 2156-2162. https://doi.org/10.1128/aem.62.6.2156-2162.1996.

Norton, J.M., Klotz, M.G., Stein, L.Y., Arp, D.J., Bottomley, P.J., Chain, P.S., Hauser, L.J., Land, M.L., Larimer, F.W., Shin, M.W., & Starkenburg, S.R. (2008). Complete genome sequence of *Nitrosospira multiformis*, an ammonia-oxidizing bacterium from the soil environment. Appl Environ Microbiol, 74, 3559-72. https://doi.org/10.1128/AEM.02722-07.

Pester, M., Maixer, F., Berry, D., Rattel, T., Koch, H., Lucker, S., Nowka, B., Richter, A., Spieck, E., Lebedeva, E., Loy, A., Wagner, M., & Daims, H. (2014). NxrB encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing *Nitrospira*. Environ Microbiol, 16, 3055–3071.

Picone, N., Pol, A., Mesman, R., et al. (2021). Ammonia oxidation at pH 2.5 by a new gammaproteobacterial ammonia-oxidizing bacterium. ISME J, 15, 1150–1164. https://doi.org/10.1038/s41396-020-00840-7.

Pjevac, P., Schauberger, C., Poghosyan, L., Herbold, C.W., van Kessel, M.A.H.J., Daebeler, A., Steinberger, M., Jetten, M.S.M., Lücker, S., Wagner, M., & Daims, H. (2017). AmoA-targeted polymerase chain reaction primers for the specific detection and quantification of comammox *Nitrospira* in the environment. Front Microbiol, 8, 1508. https://doi.org/10.3389/fmicb.2017.01508.

Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M.C., Koops, H.P., & Wagner, M. (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: Implications for molecular diversity surveys. Appl Environ Microbiol, 66, 5368–5382. https://doi.org/10.1128/AEM.66.12.5368-5382.2000.

Purkhold, U., Wagner, M., Timmermann, G., Pommerening-Röser, A., & Koops, H.P. (2003). 16S rRNA and amoA-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: Extension of the dataset and proposal of a new lineage within the nitrosomonads. Int J Syst Evol, 53, 1485-1494. https://doi.org/10.1099/ijs.0.02638-0.

Rice, M.C., Norton, J.M., Valois, F., et al. (2016). Complete genome of Nitrosospira briensis C-128, an ammonia-oxidizing bacterium from agricultural soil. Stand Genomic Sci, 11, 46. https://doi.org/10.1186/s40793-016-0168-4.

Rotthauwe, J.H., Witzel, K.P., & Liesack, W. (1997). The ammonia monooxygenase structural gene amoA as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol, 63, 4704–4712. https://doi.org/10.1128/aem.63.12.4704-4712.1997.

Schramm, A., Larsen, L.H., Revsbech, N.P., Ramsing, N.B., Amann, R., & Schleifer, K.H. (1996). Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Appl Environ Microbiol, 62, 4641-4647. https://doi.org/10.1128/aem.62.12.4641-4647.1996.

Sedlacek, C.J., McGowan, B., Suwa, Y., et al. (2019). A physiological and genomic comparison of Nitrosomonas Cluster 6a and 7 ammonia-oxidizing bacteria. Microb Ecol, 78, 985–994. https://doi.org/10.1007/s00248-019-01378-8.

Shinn, M.B. (1941). Colorimetric method for determination of nitrite. Ind Eng Chem, 13, 33–35.

Skinner, F.A., & Walker, N. (1961). Growth of *Nitrosomonas europaea* in batch and continuous culture. Arch Mikrobiol, 38, 339–349. https://doi.org/10.1007/bf00408008.

Tourna, M., Freitag,T.E., Nicol, G.W., Prosser, J.I. (2008). Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environ Microbiol. 10, 1357-64. https://doi.org/10.1111/j.1462-2920.2007.01563.x.

Tourna, M., Stieglmeier, M., Spang, A., Könneke, M., Schintlmeister, A., Urich, T., Engel, M., Schloter, M., Wagner, M., Richter, A., & Schleper, C. (2011). *Nitrososphaera viennensis*, an ammonia-oxidizing archaeon from soil. PNAS, 108, 8420-8425. https://doi.org/10.1073/pnas.1013488108.

Vanparys, B., Spieck, E., Heylen, K., Wittebolle, L., Geets, J., Boon, N., & De Vos, P. (2007). The phylogeny of the genus *Nitrobacter* based on comparative rep-PCR, 16S rRNA and nitrite oxidoreductase gene sequence analysis. Syst Appl Microbiol, 30, 297–308.

Vasileiadis, S, Puglisi, E, Papadopoulou, E S, Pertile, G, Suciu, N, Pappolla, R A, Tourna, M, Karas, P A, Papadimitriou, F, Kasiotakis, A, Ipsilanti, N, Ferrarini, A, Sułowicz, S, Fornasier, F, Menkissoglu-Spiroudi, U, Nicol, G W, Trevisan, M, Karpouzas, D G (2018). Blame it on the metabolite: 3,5-dichloraniline rather than

the parent compound is responsible for decreasing diversity and function of soil microorganisms. Appl Environ Microbiol 84, e01536-01518, 10.1128/aem.01536-18

Vasileiadis, S, Perruchon, C, Scheer, B, Adrian, L, Steinbach, N, Trevisan, M, Plaza-Bolaños, P, Agüera, A, Chatzinotas, A, Karpouzas, D G (2022). Nutritional inter-dependencies and a carbazole-dioxygenase are key elements of a bacterial consortium relying on a *Sphingomonas* for the degradation of the fungicide thiabendazole. Environ Microbiol 24, 5105-5122, https://doi.org/10.1111/1462-2920.16116

Winogradsky, S. (1890). Sur les or auismes de la nitrification. Comptes Rendus de l'Académie, 110, 1013-1016.

Zhao, Z., Huang, G., He, S., Zhou, N., Wang, M., Dang, C., Wang, J., & Zheng, M. (2019). Abundance and community composition of comammox bacteria in different ecosystems by a universal primer set. Sci Total Environ, 691, 146-155. https://doi.org/10.1016/j.scitotenv.2019.07.131.