PhD Summer School

"Biological Nitrification Inhibition: Integrating Microbial Functions, Plant Traits, and Technological Innovations for Sustainable Nitrogen Cycling"

University of Thessaly
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Organized by:

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

Practical Training

"Fast-track, high-throughput screening for BNIs in ammonia-oxidizing bacteria"

UNIVERSITY OF THESSALY

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Contents

Introduction

This practical session provides an introductory overview of a fast-track assay system developed to screen pure compounds and plant-derived root exudates for their biological nitrification inhibition (BNI) activity against ammonia-oxidizing microorganisms (AOM)[1]. The assay includes multiple soil-relevant AOM strains; in this session, we demonstrate the use of *Nitrosospira multiformis* (ATCC25196) as a model ammonia-oxidizing bacterium (AOB).

Overview of the fast-track pipeline

Once *N. multiformis* cultures reach the late logarithmic growth phase, the fast-track assay is conducted to estimate the EC_{50} values (Effective Concentration 50) of the test samples. The pipeline comprises three main stages (see Figure 1):

1) Cell harvesting and inoculation

2) Cell plating and sample application

3) Colorimetric nitrite quantification

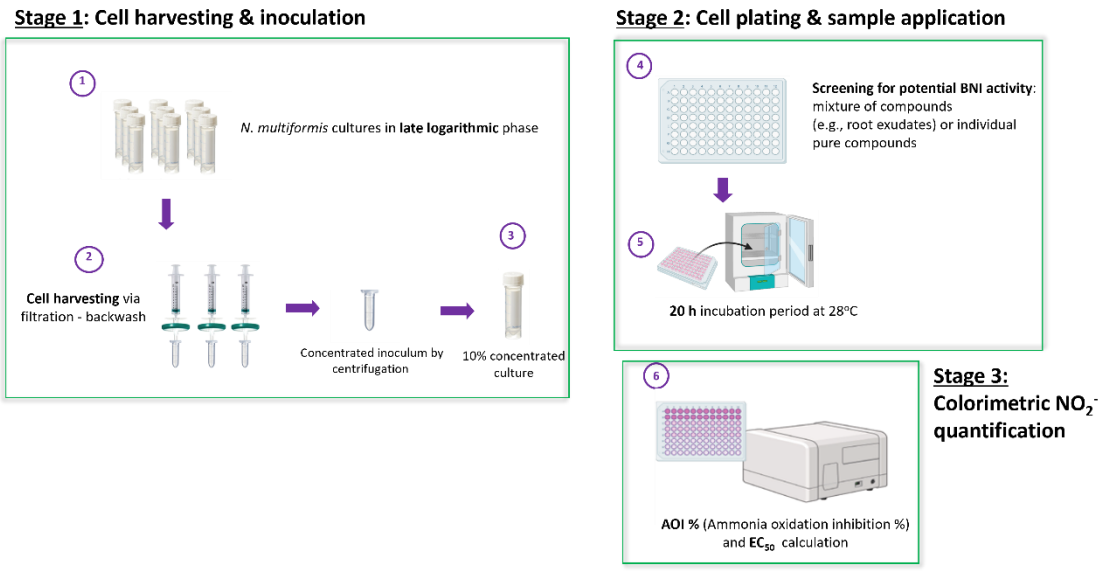

***Figure 1**. Overview of the three stages of the fast-track assay.*

[1] Kanellopoulos et al. 2024; BioRxiv. https://doi.org/10.1101/2024.12.03.626636).

In this practical training session, you will focus on stages 2 and 3 of the fast-track protocol assay. Specifically, you will: (a) prepare the "fast-track" plate, and (b) set up the plate for colorimetric nitrite quantification.

Stage 2: Plate setup for sample application

Required materials:

- One 96-well microtiter plate
- A pre-prepared 10% (v/v) concentrated culture of *N. multiformis* in Skinner-Walker (SW) medium[2]
- **ddH_2O control:** 1% (v/v) initial stock solution (diluted in SW medium)
- **DMSO control:** 1% (v/v) initial stock solution (diluted in SW)
- **Methyl 3-(4-Hydroxyphenyl) propionate (MHPP)** working solutions: 1% v/v DMSO initial stock solution with final concentrations in SW medium of: 0.55, 1.1, 2.75 and 5.5 mM
- **Hydrophobic root exudate working solutions:** 1% DMSO initial stock solution with final concentrations in SW medium of 0.22, 0.55, 1.1 and 1.65 μg μL^{-1}

Procedure:

1. **Inoculum Dispensing**

Dispense 200 μL of the 10% (v/v) *N. multiformis* concentrated culture into each well of rows B to D of the 96-well plate, as shown in Figure 2.

2. **Application of samples and controls (in triplicate)**

- **Negative controls:**

Add **20 μL of 1% (v/v) ddH_2O control** into wells **B1-B3** and **20 μL of 1% (v/v) DMSO control** into wells **B4-B6.**

- **Positive controls:**

Add **20 μL of MHPP working solutions** at the following final concentrations: **50, 100, 250 and 500 μM,** distributed across wells **C1-C12.**

- **Root exudate samples:**

Add **20 μL** of **hydrophobic root exudate working solutions** at final concentrations: **0.02, 0.05, 0.10, 0.15 μg μL^{-1},** distributed across wells **D1-D12**.

- **Incubation**

Cover the plate with a sterile lid and incubate at **28°C for 20 hours.**

[2] Skinner FA, Walker N. 1961; Arch Für Mikrobiol. 38(4):339–49.

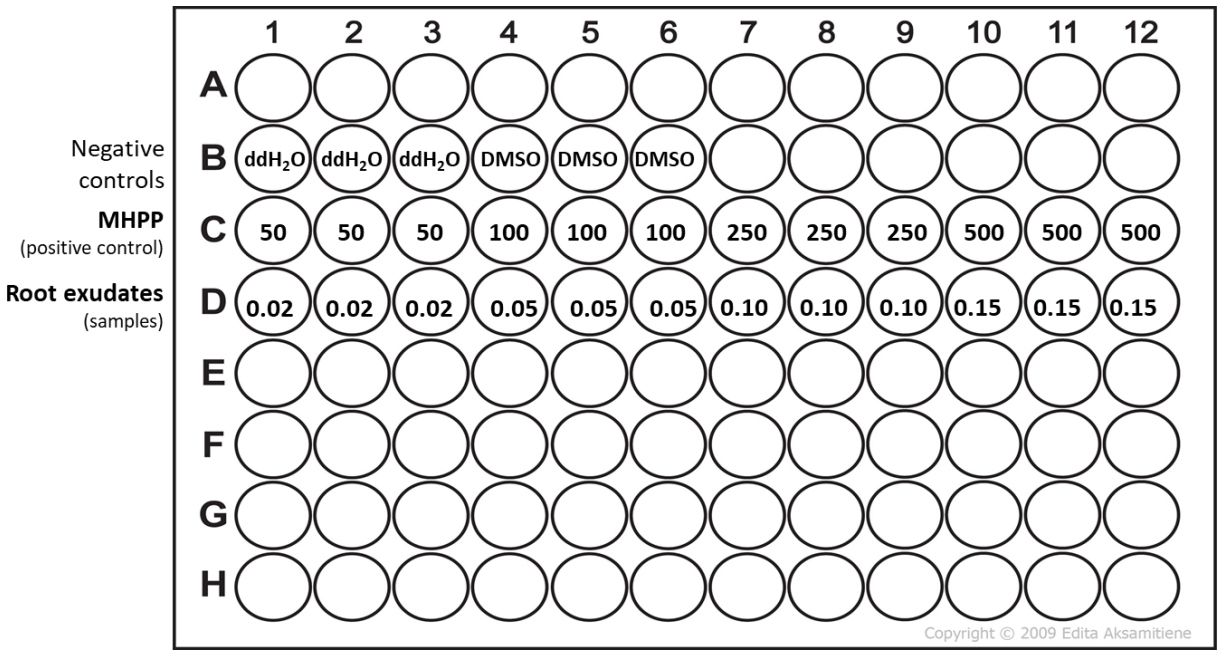

***Figure 2**. Plate setup for sample application.*

Note:

- **The final concentration of DMSO in each well will be 0.09%, a level not expected to inhibit the activity of the strain.**
- Rows A and E–H are not used in this assay.

Stage 3: Plate setup for colorimetric nitrite measurements

Following the incubation period, nitrite production is quantified using the Griess reaction[3], a colorimetric assay based on a two-step chemical process:

1. **Diazotizing reaction:** Nitrite reacts with **sulfanilamide** under acidic conditions to form a diazonium salt.
2. **Coupling reaction:** The diazonium salt then couples with **N-(1-naphthyl)ethylenediamine dihydrochloride (NED)** to form a **stable pink azo dye**.

The intensity of the resulting color is **directly proportional to the nitrite concentration** in the sample and is measured **spectrophotometrically at 540 nm.**

To perform this stage, you will be provided with **pre-incubated "fast-track" assay plates**, prepared identically to those assembled in Stage 2. These will be used for the colorimetric detection of nitrite accumulation, reflecting the inhibitory or stimulatory effects of the tested compounds on *N.*

[3] Shinn MB. ACS Publications. American Chemical Society; 1941. https://pubs.acs.org/doi/abs/10.1021/i560089a010

multiformis activity.

Required materials:

- **Incubated 96-well plate** from Stage 2 (containing the test samples)
- **One fresh 96-well microtiter plate** (for colorimetric measurement)
- **Sodium nitrite ($NaNO_2$) standard solutions** at the following concentrations: 0, 1, 2, 5, 10, 20, 50, 100 and 200 µM
- **Skinner and Walker (SW) medium** in a **30 mL sterile polystyrene Sterilin™ bottle**
- **Diazotizing reagent** (typically contains sulfanilamide in acidic solution)
- **Coupling reagent** (typically contains N-(1-naphthyl)ethylenediamine dihydrochloride, NED)

Procedure:

1. **Preparation of standard curve**

   Add 100 µL of $NaNO_2$ standard curve solutions in the range: 0, 1, 2, 5, 10, 20, 50, 100 and 200 µM into wells A1 to A9 of the 96-well plate to establish the nitrite standard curve.

2. **Sample dilution**

   Dispense 90 µL of SW medium into the appropriate wells of rows B to D (see Figure 3). Then, transfer 10 µL of each sample from the pre-incubated assay plate (from stage 2) into the corresponding wells of the new plate (rows B-D). This results in a 1:10 dilution of each sample to ensure compatibility with the standard curve range.

3. **Color development via Griess reaction**

   Add **20 µL of diazotizing reagent** to each well, followed by **20 µL of coupling reagent**. A magenta color will develop immediately in wells containing nitrite, with **color intensity directly proportional to the nitrite concentration.**

4. **Incubation**

   Incubate the plate for **10 minutes at room temperature** to allow full color development.

5. **Nitrite measurement**
   Measure the **absorbance at 540 nm** using a microplate reader.

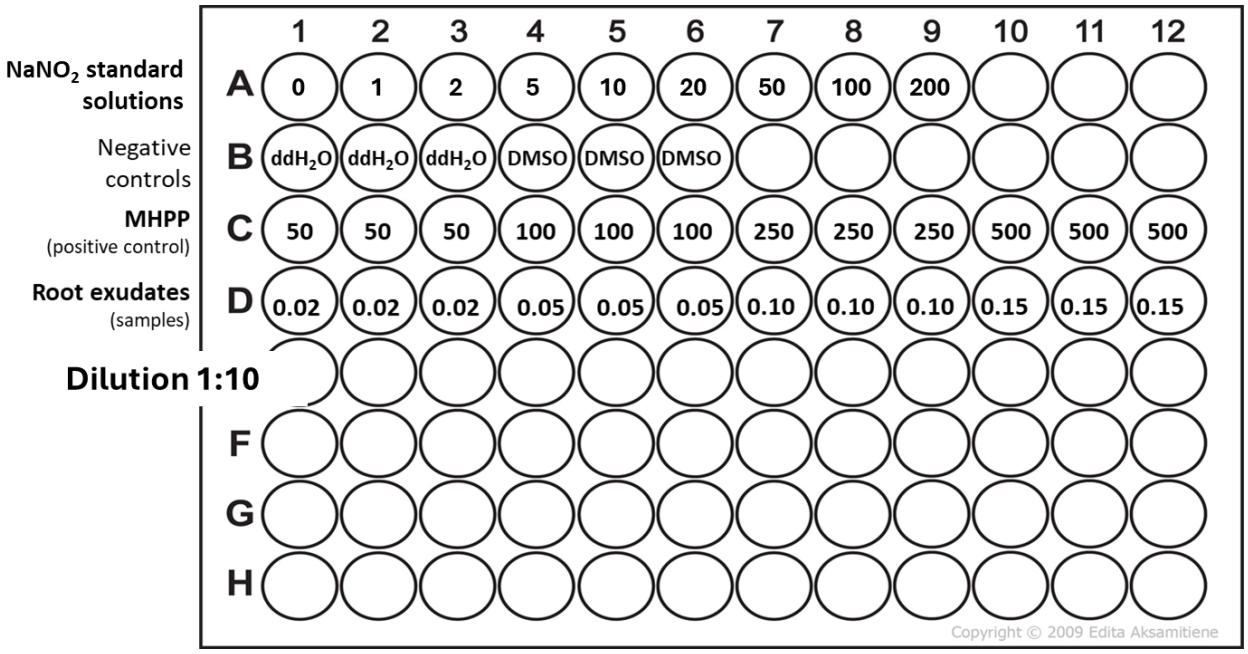

***Figure 3**. Plate setup for the nitrite measurements*

Data Processing

1. Normalize nitrite values

For each sample and control, normalize the nitrite concentration by dividing by the average value of the negative controls:

$$\boldsymbol{Normalised\ NO_{2_{control}}} = \frac{NO_{2_{control}}}{Average\ NO_{2_{control}}}$$

$$\boldsymbol{Normalised\ NO_{2_{sample}}} = \frac{NO_{2_{sample}}}{Average\ NO_{2_{control}}}$$

2. Calculate Ammonia-Oxidation Inhibition (AOI%)

For each replicate, calculate the AOI% based on the deviation from the control, following the equation:

$$\boldsymbol{AOI}\ \% = -(Normalised\ NO_{2_{sample}} - Normalised\ NO_{2_{control}})$$

3. Plot the AOI% vs. Concentration

Use the AOI% values for each tested concentration to generate inhibition curves. These plots can be used to estimate the EC_{50} (Effective Concentration at which 50% inhibition is observed), using curve-fitting methods (e.g., non-linear regression).