Experiment	**Nitrate as alternative electron acceptor: Denitrification**
Advisor	Christine Lehmann chleh@botinst.unizh.ch phone: 01/634 82 81
Reading	Chapters in BBOM 9th: 15.15 (Anaerobic Respiration), 15.16 (Nitrate Reduction, Denitrification Process), 16.16 (The Nitrogen Cycle), BBOM 9th: Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 9th Edition, Prentice Hall, 1999.
Objectives	• Understand denitrification as an anaerobic respiration process and the bacteria capable of doing it. • Practical work: Study and test a given bacterium or an environmental sample for dissimilatory nitrate reduction: 1) Nitrate reduction to nitrite 2) Nitrite reduction to N_2O and N_2
Background	In addition to their assimilatory uptake by plants and microbes, inorganic nitrogen compounds can serve as electron acceptors under anoxic or oxygen-depleted conditions during dissimilation. Nitrate or nitrite is used as an alternative oxidant at the expense of an organic or inorganic electron acceptor in anaerobic respiration. They are eventually converted to NO, N_2O and N_2. This process is called denitrification (Fig. 1). It is characterized as follows: • The products of dissimilatory denitrification are excreted into the environment. • The most important enzyme involved in this process is the nitrate reductase which is membrane-bound. • Energy is generated via electron transport processes in the bacterial membrane. • *Pseudomonas* species, for example, are well-known nitrate reducers. • Bacterial species may be differentiated on the basis of their ability to reduce nitrate to nitrite or to nitrogenous gases. • In medical microbiology, the denitrification test is diagnostic for the identification of enteric bacteria. Fig. 1: Denitrification processes **Redox state** +V NO_3^- **Nitrate** +III NO_2^- **Nitrite** +II **NO** **Nitric oxide** +I **N_2O** **Nitrous oxide** 0 N_2 **Nitrogen gas** $NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O$ pH↑ $2NO_2^- + 6e^- + 8H^+ \rightarrow N_2 + 4H_2O$ pH↑ N_2O acts as a greenhouse gas. In the stratosphere it is converted to NO which contributes to the destruction of the ozon layer. For the environmental impact of denitrification see http://www.epa.gov/globalwarming

Literature

- Nicholas, D.J.D. and A. Nason. 1973. Nitrite determination by diazotization and coupling reactions. In: Colowick S.P. and NO Kaplan (eds) Methods in Enzymology, vol. III, Academic Press; New York, pp 983-984.
- Parham, N.J.A. and G.R. Gibson. 2000. Microbes involved in dissimilatory nitrate reduction in the human large intestine. FEMS Microbiology Ecology 31:21-28.
- Christensen, P.B., Rysgaard, S., Sloth, N.P., Dalsgaard, T. and S. Schwaerter. 2000. Sediment mineralization, nutrient fluxes, denitrification and dissimilatory nitrate reduction to ammonium in an estuarine fjord with sea cage trout farms. Aquatic Microbial Ecology 21:73-84.

www. Links

http://www.bsi.vt.edu/chagedor/biol_4684/Cycles/Denit.html

Practical Work

The students will:

- Create nitrate-reducing conditions in a test tube and handle pure cultures and environmental samples.
- Perform a biochemical test for denitrification.
- Write a short laboratory report.

Materials and Experimental Protocols

Glassware and other materials per student group

4 culture tubes (16 mm x 105 mm) with butyl rubber stoppers (or screw-cap test tubes), 4 'Durham' tubes (6 mm x 35 mm, heavy-walled), 4 test tubes (16 mm x 105 mm), 1 inoculating loop, 2 racks, pipettes (200 µl, 1 ml, 5 ml), pipette tips, tape, waterproof pen, incubator (32 °C), spectrophotometer

Nitrate Reduction Broth (components in g/l, prepared)

Meat extract 1, Yeast extract 2, Peptone 5, NaCl 5, KNO_3 2, pH 7.

Test organisms: Pure cultures and environmental samples.

Reagents for Nitrite test (prepared)

- Nitrite test solution: mix equal volumes of solutions A and B to make the test reagent
  Solution A: **Sulfanilic acid (diazotizing reagent):** Dissolve 1 g 4-aminobenzene sulfonic acid in 100 ml 1.5 N HCl solution by gentle heating; store at room temperature, protected from light in an amber bottle.
  Solution B: **Alpha-Naphthylamine solution (coupling reagent)**: Dissolve 0.02 g N-(1-Naphthyl)ethylenediamine dihydrochloride in 100 ml 1.5 N HCl solution by gentle heating in a fume hood; store refrigerated.
- Zinc powder

INOCULATION PROCEDURE

1. Dissolve the ingredients of the Nitrate Reduction Broth in distilled water; adjust solution to pH 7.0 (prepared)
2. Prepare 4 tubes with nitrate broth: dispense 5-ml aliquots of the broth into tubes
3. Insert an inverted ‚Durham' tube into each tube
4. Autoclave the tubes for 15 min at 121°C
5. Store medium at 4°C until used
6. Label each tube (# 1 to 4) with the date and the name of the microorganism to be inoculated
7. Prewarm the medium to room temperature before inoculation
8. Invert tubes in order to release all the air still present the Durham tube
9. Using aseptic techniques, inoculate 500 µl of each microorganism or environmental sample into its appropriately labeled tube
10. The fourth tube will be the uninoculated control
11. Check pH; it should be 7!
12. Incubate the cultures for 2-7 days at 32 °C.

NITRITE TEST PROCEDURE

The microorganism's ability to reduce nitrate to nitrite is determined by the addition of the diazotizing and the coupling reagent to the test tube

1. Label 4 assay tubes (# 1to 4) and add 4 ml distilled H_2O to each
2. Add 20 µl aliquotes from each culture to the appropriate assay tube
3. Add 2 ml nitrite test solution to each assay tube
4. Allow the color to develop for 15 min
5. The intensity of the purple-red color is measured as absorbance at 540 nm in a photometer
6. Compare with an appropriate standard calibration

Results: In case of a **positive reaction,** you will immediately observe a purple-red color in the test tube; i.e. the bacteria are positive for nitrate reduction and produced nitrite. The positive reaction can be written as follows:

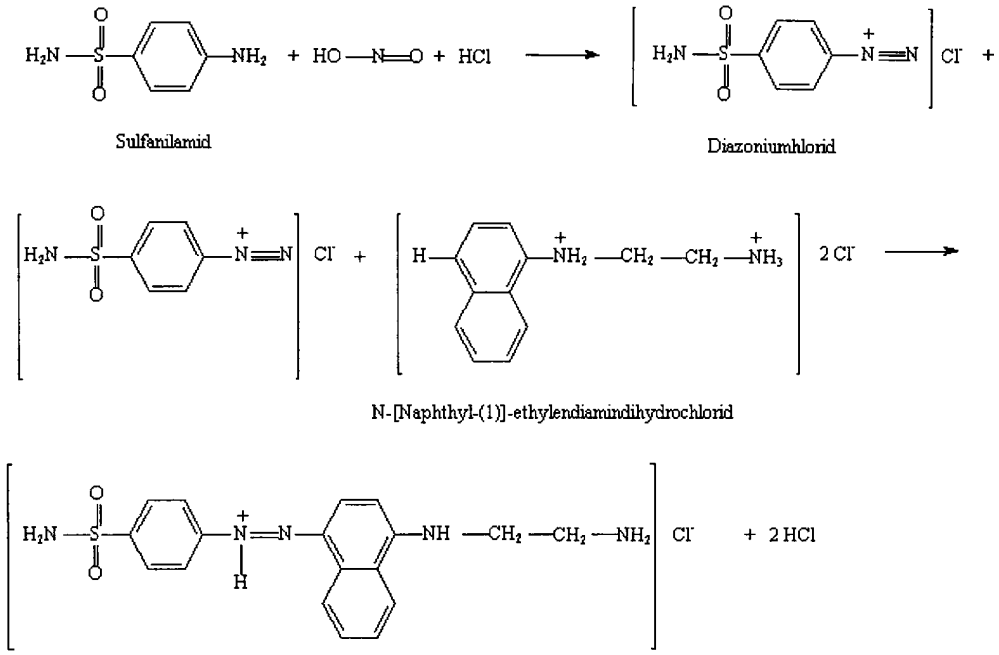

Fig. 2: Intermediate and end-products of nitrite test
Source: http: www.uni-bayreuth.de/departments/ddchemie/wurst/abb9.gif.

In case of an apparently **negative reaction** (no nitrite detected, i.e., the suspension is colorless after the addition of the Nitrite test reagent), further testing must be done.

IS THERE STILL NITRATE PRESENT IN THE CULTURE MEDIUM?

Addition of zinc powder: chemical reduction of nitrate to nitrite

1. Add a small amount ("sharp knife point": ca. 5 mg) of zinc dust to the test tube that tested negative for nitrite production (nitrite test reagent is present in the tube).
2. Shake the tube vigorously and allow it to stand at room temperature for 10-15 min.

Result:

- If the medium remains colorless, the test result for nitrate reduction is **positive** (i.e. bacterial nitrate reduction to gaseous compounds took place; nitrate was used up and transformed with no nitrate or nitrite remaining).
- If the medium turns red after the addition of Zn powder, the test result for nitrate reduction is **negative**; nitrate was not used by the microorganisms. It was chemically reduced to nitrite by the test reagent.

TEST FOR NITROGENOUS GASES (N_2 and N_2O)

Observe the inverted Durham tubes for gas production.
http://www.cat.cc.md.us/~gkaiser/labmanua/lab8/prgas.html

Laboratory Rules & Precautions

- **α-Naphthylamine** is a **carcinogen**. On the basis of structural similarity, N-(1-Naphthyl)ethylenediamine might also be dangerous. That's why all precautions pertinent to carcinogens should be observed for this compound. Do not use a mouth-drawn pipet. Wear gloves. Dispose of waste properly (ask assistant).
- **HCl** is **corrosive**. Contact with skin may cause blisters and burns. In case of contact, flush immediately with plenty of water (for at least 15 min.)

Goals & Experiences gained

Microbial analyses:
The nitrate reduction test is based on the detection of nitrite and its ability to form a red compound when it reacts with sulfanilic acid to form a complex (nitrite-sulfanilic acid) which then reacts with alpha-naphthylamine to give a red azo dye; gases are trapped in the inverted Durham tube.

Timing

60 min.

Reporting

Measure turbidity, perfom the nitrite tests, observe the organisms in the microscope and report your results!

LABORATORY REPORT: Nitrate Reduction
Date:
PURPOSE: . . . . . .

. . . . . .

. . . . . .

Sample No.	‚turbidity' (OD_{660nm})	Nitrite produced + / -	Gas produced + / -	Nitrate left in the medium	Types of microbes observed
1					
2					
3					
4					

Questions to be answered

1. What do the gas bubbles in the Durham tube tell you? Why is gas production without gas analysis not sufficient to claim that denitrification took place ?
2. How can you avoid growing fermenting bacteria in an enrichment culture for denitrifiers ?
3. Answer the questions: http://www.smccd.net/accounts/case/biol240/NO.html
4. For problems see: Section Problems & Solutions under http://www.cdc.gov/ncidod/dastlr/gcdir/NeIdent/Nitrate.html
5. How could you make the nitrate reduction test a quantitative one?
6. Formulate incomplete denitrification to N_2O and NO as stoichiometrically balanced equations; use methanol as the electron donor.
7. Name a few bacteria which are denitrifyers. Does your list contain any obligate denitrifyers or are they all facultative ones ?
8. Analogously, sulfate can act as an alternative electron acceptor for certain anaerobes. Name a few sulfate reducers and formulate a sulfate-reducing redox reaction with an appropriate electron donor substrate.