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Protocol

DBNSOP06

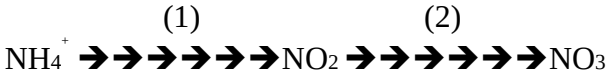
NITRIFYING BACTERIA, N-BART™

Background

Ammonium is a common product of the breakdown of organic nitrogen under anaerobic (oxygen-free) conditions. The nitrifying bacteria oxidize the ammonium to nitrate in soils and waters. This nitrate now becomes a hazard if it builds up in the water. This test is a different form of BART test in that it has a fixed testing period and involves the detection of nitrite as an indicator of the presence of nitrifying bacteria. Nitrate is not used in this detection system because the amounts of nitrate can be transient particularly when there is a considerable amount of denitrification occurring at the same time. Unlike other tests, a reaction cap is provided that is used to allow the detection of the nitrite after a five-day incubation period. A positive reaction occurs when a pink to red coloring is produced in the tester within thirty minutes of the reaction cap being applied. A negative detection would occur when no color reaction is produced in the tester.

Theoretical

Nitrification serves as the major route by which ammonium is aerobically oxidized to nitrate. There are two steps to nitrification process:



Ammonium Nitrite Nitrate

Nitrifying bacteria are divided according to which of the above reactions (1 or 2) they are able to perform:

Group 1 -step (1) only - Nitrosofiers ➔ *Nitrosomonas*
Group 2 -step (2) only - Nitrifiers ➔ *Nitrobacter*

The polarized relationship between the nitrifying and the denitrifying bacteria is a problem in the testing of natural samples since the two both groups are either producing or utilizing nitrate respectively. In developing a test system for the nitrifying bacteria in natural samples, the terminal product (nitrate) may not be recoverable because of the intrinsic activities of the denitrifying bacteria which are also likely to be present and active in the sample. It is because of this difficulty that the N-BART tester restricts itself to detecting the nitrosofiers that generate nitrite. This nitrite will be oxidized to nitrate by the nitrifiers only to reappear when reduced back to nitrite by any intrinsic denitrification occurring in the sample.

The nitrifying bacteria are an important indicator group for the recycling of organic nitrogenous materials from ammonium (the end point for the decomposition of proteins) to the production of nitrates. In waters, the presence of an aggressive population if nitrifiers are taken to indicate that there is a potential for significant amounts of nitrate to be generated in the waters which are aerobic (rich in oxygen). Nitrates in water are a cause of concern because of the potential health risk particularly to infants who have not yet developed a tolerance to nitrates. In soils, nitrification is considered to be very significant and useful functions in the recycling of nitrogen through the soil. Nitrate is a highly mobile ion in the soil and will move (diffuse) relatively quickly while ammonium remains relatively "locked" in the soil. In some agronomic practices, nitrification inhibitors have been used to reduce the "losses" of ammonium to nitrate.

A common use for the presence of active nitrifying bacteria in waters is that these bacteria signal the latter stages in the aerobic degradation of nitrogen-rich organic materials. Aggressive presence of nitrifying bacteria in water can be used to indicate that there is a potential for the water to have been polluted by nitrogen-rich organics from such sources as compromised septic tanks, sewage systems, industrial and hazardous waste sites and is undergoing an aerobic form of degradation. Nitrification and denitrification are essentially parallel processes that function in a contradictory manner. It is recommended that, where a high aggressivity is determined, waters should be subjected to further evaluation as a hygiene risk through a subsequent determination for the presence of nitrates. In soils, the presence of an aggressive denitrifying bacterial population may be taken to indicate that the nitrification part of the soil nitrogen cycle is functional. Nitrification is fundamentally an aerobic process in which the ammonium is oxidatively converted to nitrate via nitrite. Nitrite produced by the denitrification of nitrate may also be oxidized back to nitrate.

Reaction Patterns, Nitrifying Bacteria, N-BART

This test is an unusual test in that the presence of nitrifying bacteria is detected by the presence if nitrite in the test vial after a standard incubation period of five days. Nitrification involves the oxidation of ammonium to nitrate via nitrite. Unfortunately, in natural samples, there are commonly denitrifying bacteria present in the water and these reduce the nitrate back to nitrite. If denitrification is completed, this nitrite may be reduced further to dinitrogen gas (under anaerobic conditions). That is why this test is laid upon its side with three balls to provide a moistened highly aerobic upper surface where nitrification is most likely to occur through oxidative processes. The reagent administered in the reaction cap detects nitrite specifically by a red color reaction. This test is interpreted by the amount of pink-red coloration generated, and the location of this color. There are three levels of reaction that can be observed:

PP -Pink-red color on roughly half the ball, solution clear or pale yellow (Reaction 1)

RP -All balls are reddened, solution may be pale pink (Reaction 2)

DR -Balls and the solution are reddened (Reaction 3)

This test is different to the other BART™ tests in that a chemical reagent is added to detect the product (nitrite) after a standard incubation period. The typical reactions are described below:

PP – Partial Pink on the Balls

Clear solution but a pink reaction may be generated on the BART balls indicating that nitrification has just begun and the nitrite detected is in the biofilm on the balls.

RP – Red Deposits and Pink Solution

Reaction causes a light pink solution with red deposits all over the three BART balls. Nitrite is now present in solution as well as in the biofilms on the balls.

DR – Dark Red Deposits and Solution

Reaction causes dark red solution with heavy red deposits on the BART balls. High concentrations of nitrite have been detected indicating an aggressive level of nitrification has occurred in the test period.

Interpretation of Reaction Patterns for the N-BART™

The reaction represents the population size and does not reflect the variety of microorganisms present in the water sample:

PP Small population of nitrifiers (< 10^2 nitrifiers/ml) associated with aerobic slime

forming bacteria in a consortium

RP Moderate population of nitrifiers (> 10^2 and < 10^5 nitrifiers/ml) forming a major component in the bacterial flora

DR Dominant population of nitrifiers (> 10^5 nitrifiers/ml)

Hygiene Risk Considerations

The presence of an aggressive population of nitrifying bacteria in water is taken to indicate that there is a potential for significant amounts of nitrate to be generated in waters which are aerobic in nature. This may indicate a potential health risk particularly to infants who have not yet developed a tolerance to nitrates. It is recommended that, where a high population is determined, waters should be subjected to further evaluation as a hygiene risk, through subsequent determination for the presence of nitrates.

This test detects the nitrifying bacteria that are able to oxidize ammonium (NH_4) to nitrite (NO_2) and on to nitrate (NO_3). This test uses a selective medium for the bacteria able to oxidize ammonium to nitrite by examining chemically for the nitrite product. The additional two BART balls used in this test provide larger solid surfaces in contact: with the air on the upper hemispheres of the three balls in the tester. This encourages nitrification in the liquid film over the balls. In the early stages, the first (product) nitrite is detected at these sites. A reactant cap is used to detect the presence of nitrite that is generated during the early stages of nitrification. If the sample being tested also contains denitrifying bacteria, nitrite may again be created by the reduction of nitrate (denitrification). This test method has been developed in consideration of the greater likelihood of nitrite being detectable rather than the (product) nitrate. Note that this test cannot function in water samples with a natural nitrite level of greater than 3.0 ppm. Water samples with greater than 28 ppm of nitrite will automatically turn the liquid medium to a yellow color when the reaction cap test is applied.

N-BART™ Selective medium

Circular white crystalline opaque deposit remains clustered around the central peg in the basal cone of the tester. This extension may be 5 to 8 mm in radius with a defined largely smooth edge. In the normal event of the confirmation of sterility, there is a change in the characteristics of the liquid medium forming in the test vial.

N-BART Reaction Cap.

This cap is a small screw type white plastic cap which can be screwed down onto the inner test vial. This cap contains rough porous paper disc fitted within the inner flange. When viewed from the under side, this disc is colored. It varies from a solid pink to a yellowish pink center with a darker pink perimeter. This color is generated by the reactants used to detect nitrite in the test medium. While there is a variation in the color, it has been found that this does not affect the accuracy of the test method. Controls

applied under sterile conditions will show that the basal medium will dissolve by twelve hours leaving the sterile liquid in the tester crystal clear. Natural water samples can cause minor chemical reactions which may be seen through an intensification of the color at the diffusion front with occasionally crystalline deposits forming in the along the floor of the tester.

The N-BART tester is a relatively specialized test in which the medium will not support a wide range of contaminants. Where there is contamination, the initial expression of growth is a light clouding (day 1) which gradually intensifies causing the medium to go turbid. If there are any complete denitrifiers among the contaminants, gassing may occur.

Confirmation of the Selective Media Composition in the N-BART™

In order to confirm the suitability of the selective medium for the tester of the various bacteria recognized by this test method, it is recommended that the following A.T.C.C. (American Type Culture Collection) strains be applied to the N-BART™ testers to determine the standard reaction patterns. Each culture should be prepared as a 7-day culture incubated at 25°C to reach the stationary growth phase. The cultural techniques to be used are referenced in Verhagen et al (1993) "Effects of Grazing by Flagellates on Competition for Ammonium between Nitrifying and Heterotrophic Bacteria in Soil Columns" J. Appl. and Appl. Micro. Inoculation of the inner test vial should be with a 0.1ml suspension of the broth culture in 15 ml of the sterile Ringer's solution. This inoculum should be taken from the midpoint of the broth culture immediately after the culture had been gently agitated. 7.75 ml of this inoculated solution should be applied directly to the tester. Do not shake the vial. Incubate at 22 to 24°C for five days and observe for activities and reactions after applying the reactant cap following the standard procedure. Typical results are listed below for the recommended A.T.C.C. strains for confirming the effectiveness of the N-BART tester: *Nitrosomonas winogradski*25391 will show clouding, red reaction, nitrite +; *Pseudomonas aeruginosa* 27853 will show no reaction, nitrite negative; and *Nitrosomonas europae* 19718 will show clouding, red reaction, nitrite +. In the event that ATCC cultures are not available then a SE or FE sample from a municipal waste water treatment plant can be used if the treatment method is aerobic. The high ammonium content commonly in PE usually triggered nitrification in the secondary and tertiary treatments. Note the sample should be taken before any final disinfection treatment (such as U.V.) is applied.

QC Procedure for the N Reactant Cap

Prepare or obtain analytical grade solutions of sodium nitrite stock solution and dilute standard sodium nitrite solution (5mg and 50mg $N\text{-}NO_2/L$). These analytical solutions should be labelled nitrite 5 and nitrite 50 and should be freshly prepared using sterile distilled water and aseptic techniques. The protocol for testing the reactant cap would be similar for all solutions. This protocol would involve the following steps:

A. Sodium nitrite stock solution (**solution A**) Dissolve 100mg $NaNO_2$ in 50ml distilled water. Transfer it to a 200-ml volumetric flask. Add distilled water to

mark line. Final concentration is 500mg/L.

B. Dilute standard $NaNO_2$ solution:

Solution nitrite 5: Pipette 0.5ml **solution A** to a 50-ml volumetric flask. Add distilled water to mark line. Final concentration is 5mg/L.

Solution nitrite 50: Pipette 5.0ml **solution A** to a 50-ml volumetric flask. Add distilled water to mark line. Final concentration is 50mg/L.

Prepare the solutions immediately before use.

C. Prepare three tubes. Put one white ball in each tester vial. Pipette 15mls of solution nitrite 5 into each tube. Prepare another three vials. Put one white ball in each tube. Pipette 15mls of nitrite 50 into each tube. Screw on D-RX cap. Turn over onto caps for 30 minutes. Turn back and observe the colour change after 3 hours. For the tube with **nitrite 5**, the solution colour will be pink. For the tube with **nitrite 50**, the solution colour will be yellow.

Note: In weak reactions for the nitrite solutions, a weakened reaction giving lower color generation and absorption values suggests a degenerating reactant cap.

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Take one N-BART tester and place on the work top. Look for the reaction caps tube which should be present in the box of seven testers. There would be seven reaction caps within the tube if none have yet been used. Note where the reaction cap tube have been placed because a reaction cap will be needed when the tester has been incubated for five days to determine whether nitrite has been produced during the incubation period.

1. To begin the test, remove one the N-BART field testers from its foil pouch and placed upright on a clean dry bench. Unscrew the outer cap and lift the inner vial (containing the three BART balls) from the outer vial and place on a clean dry surface.
2. Unscrew the inner cap and pipette 7.75ml of the water sample to be tested.
3. Screw the inner cap back down in the inner vial and place the inner vial back into the outer vial and screw down the outer cap.
4. Place the tester on its side so that each of the BART balls is only partially immersed in the sample being tested. Leave at room temperature for five days.
5. After five days, remove the inner vial from the outer vial and now keep the inner vial upright.
6. Remove one reaction cap from the reaction cap tube.
7. Unscrew and remove the inner cap from the inner tester vial and replace with a reaction cap which should be screwed down firmly.
8. Invert the inner test vial now that the reaction cap has been placed on the vial for thirty minutes. It is recommended that the tube should be placed upside down on a clean dry surface away from any risk of being knocked over.

9. After 30 minutes of inversion then the tube is returned to vertical (cap side up) and left for 150 minutes to allow the color reactions to develop.
10. 180 minutes after placing the reaction cap on the inner vial and inverting, examine the tester for pink or red colorations and assign the reaction code (PP, RP, DR or not detected).

Disposal methods

N-BART testers, when charged with a sample and incubated, are likely to contain active bacterial populations whether the tester has gone positive for acid producing bacteria or not. Such testers may be used for confirmatory tests in a certified microbiology laboratory but most would then be disposed off since they are single use disposable test methods. Disposal may vary with the location of the completed testers. In the laboratory setting, the testers should be placed in a biohazard bag which would then be sealed prior to steam or gas sterilization. Once sterilized then the testers do not present a health risk issue and should be disposed of with the regular laboratory solid wastes.

In the event that the testers have been used at too great a distance from a suitable certified microbiology laboratory or do not any arrangement to get the samples to such a laboratory then the testers do have the potential to contain active bacterial cultures. To eliminate the hygiene risks from these bacteria to the general society through disposal as domestic garbage, the testers need to be disinfected or pasteurized prior to final disposal. Recommended methods for this are listed below:

Disinfection of Used Testers.

Take an 10.5” x 11.25”(27 x 28.5 cms) plastic freezer bag that has a double closure (zip lock) that can securely open and close the bag. Open the bag and place six 8.5” x 11” (22.5 x 29 cms) sheets of household paper towel which have been folded along the longer side of the sheet to make a “v” shape fold. These sheets are placed in fold side down and opened so that there are six sheets on each side of the bag. Up to 9 N-BART testers can be placed in the center of the bag lying on their side (make sure the caps have been screwed down tightly onto the vials). Once the testers have been added then 125ml of household bleach is poured into the bag. This bleach is soaked up by the paper towel. The bag is now sealed and can be disposed of with domestic garbage. Note that the normal function in trash collection includes compressing the garbage which would cause the plastic vials to fracture and leak. There would be a sufficient active disinfectant in the bleach to assure the disinfection of the contents so that the risks are no greater than for the rest of the domestic garbage.

Pasteurization of Used Testers.

Heat can be employed to kill the bacteria that have grown in the tester. The recommended method here involves the use of dedicated 800 to 1,000 watt microwave that would only be used for this purpose. To perform this treatment the initial steps are the same as given above for disinfection using a plastic freezer bag and placing the finished testers inside the bag but here there are two differences: (1) the testers are set up right; and (2) the caps are not screwed down tightly. The microwave should be activated for 50 seconds for up to 9 N-BART testers. This amount of heat would be sufficient to pasteurize the contents and cause sufficient distortion in the plastic vials to allow the contents to leak out and be absorbed by the paper towel. After the heat treatment then the sealed bags can be disposed with domestic garbage.

August 13, 2006