DROYCON BIOCONCEPTS INC
315 Dewdney Avenue, Regina, Saskatchewan, Canada S4N 0E7 (306) 585 1762;
www.dbi.ca

DN-BART tester

Standard Operating Procedure

DBDSOP06

DN-BART™ is a BART tester designed to determine the activity level of denitrifying bacteria in water. This bacterial group actually reduces nitrates under anaerobic conditions to nitrogen gas. This activity is significant since it significantly reduces the nitrate content in reductive waters and this reduces the serious health concerns particularly for babies. It is available as both laboratory (L) and field (T) testers. If the tests arte to be performed away from a laboratory then it is recommended that the F testers should be used since they protect the user from odors and the risks of leakage through the use of double vials. In the laboratory setting with normal microbiological practices then the L testers are recommended since these are more economical in their use. The DBDSOP06 protocol has been designed to cover the use the testers in either field (double vial) or laboratory (single vial) format.

Introduction

This group of bacteria is called the denitrifying bacteria and the majority of these are able to reduce the nitrate to nitrogen gas. In this test, a positive detection occurs when this nitrogen gas forms foam from nitrogen gas bubbles that usually collects around the ball generally within three days but not commonly longer than five days. The presence of this foam by the end of day three is taken to be an indication of an aggressive population of denitrifying bacteria. Absence of foam, regardless of any clouding of the water, indicates that the test is negative for the detection of denitrifying bacteria. Bubbles perched on the walls of the tester or randomly attached under the ball are not considered to be a positive indication of denitrification and these events should be ignored. It is only when gas bubble form a continuous ring around the ball or form a foam that a positive detection can be considered to have occurred This test is particularly applicable to any waters where there is likely to be potential septic or organic contamination. The presence of denitrifiers in these waters would indicate a potential health risk due to either septic wastes or nitrates in the water.

Theoretical Considerations

This test detects bacteria that can reduce nitrate (NO_3) to dinitrogen gas (N_2) by the observation of gassing which occurs when the nitrate has been completely denitrified. While nitrite is an intermediate in the denitrification of nitrates (with dinitrogen gas being the terminal product), it does not remain resident for a significant period of time particularly where nitrifying bacteria are also active to be an effective indicator of activity.

Where there is an active population of denitrifying bacteria present in the sample, the liquid sample will show a variable amount of cloudiness but there will be a generation of (dinitrogen) gas bubbles. These usually collect around the ball in the form of foam. This foam will last one to three days and usually not show any color. The presence of a foam or (less commonly) gas bubbles under and around the ball covering at least 50% of the submerged area of the ball. Cloudiness without gas formation cannot be considered a positive detection of denitrifying bacteria.

DN-BART™ Selective Medium used in the tester

This medium forms an opaque beige crystalline deposit in the base which extends out towards the walls of the test vial. It has a defined but irregular edge and appears to darken somewhat towards the central peg. Occasionally, crystalline deposits may also be seen at the wall conical base interface and may extend up the sidewall of the inner test vial up to 3mm. In a control test in which bacteria are absent then the basal medium pellet remains a light yellow and there is no coloration of the water column in the tester It should be noted that for the liquid medium to remain crystal clear then it has been generated using sterile distilled or deionized water. Natural water samples can cause minor chemical reactions that may be seen through an intensification of color in the diffusion front and crystalline deposits may form in the base of the test vial. These crystalline deposits can be differentiated from a basal slime since the crystalline deposits swirl up and have a defined edge. They generally do not have a gel-like appearance, and settle rapidly to the base after shaking. Water saturated with oxygen stored at low temperatures can, when used in this test, cause bubbles to form as oxygen comes out of solution as the temperature rises to room temperature. Therefore do not use water taken directly from a refrigerated or cold source but allow the water to rise to room temperature before beginning the test to ensure any surplus saturation of the water with oxygen has vented.

The medium used in the DN-BART™ can encourage the growth of a range of facultatively anaerobic and nitrate respiring bacteria. If there has been contamination of the test vial then this test will commonly exhibit a cloudy medium that gradually becomes more turbid with time. If any of the contaminants are complete denitrifiers, gassing may occur. Incubation for this test is normally at 22 to 24°C but using blood heat (35 to 37°C) can speed up the growth of many contaminants.

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

In order to confirm the suitability of the selective medium for the detection of the various bacteria recognized by this test method (see text above), it is recommended that the following A.T.C.C. (American Type Culture Collection) strains be applied to the DN-BART testers to determine the standard reaction patterns. Each culture should be prepared as a 48-hour culture incubated at 35°C to reach the stationary growth phase using Brain Heart Infusion broth. Inoculation of the inner test vial should be with a suspension of 0.1 ml 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. This inoculated solution should be applied directly over the BART ball as the test vial is filled. Do not shake the vial. Incubate at 22 to 24°C for one day and observe for activities and reactions after applying the reactant cap following the standard procedure. Typical results for A.T.C.C. strains are listed below by A.T.C.C. genus/species growth (by plus signs) then (in brackets) the reaction on the tester medium:

13048 *Enterobacter aerogenes* ++ (clouding),

27853 *Pseudomonas aeruginosa* ++ (slight clouding)

12228 *Staphylococcus epidermidis* – (clouding)

19606 *Acinetobacter calcoaceticus* ++ (no clouding)

25922 *Escherichia coli* ++ (clouding)

Note that gassing or foaming (++) is considered to be the prime test for complete denitrification that can be recognized as a foam ring or intense bubbles under and around the ball. Clouding is not a confirmation of denitrification and should be considered negative.

In the event that ATCC strains are not available then either PE or SE samples from a municipal waste water treatment plant can be used with an FO reaction being achieved by day 3 in PE and day 5 in SE depending upon the nature of the treatment being applied.

Uses for the DN-BART™ tester

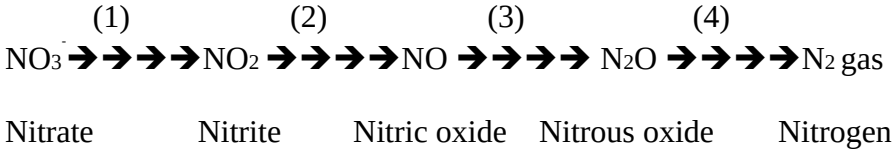
DN is short for denitrification. This activity is extremely important not only in environmental but also in geochemical terms. The reason for this is that essentially all of the atmospheric nitrogen (N_2) has been derived from the process of denitrification which is driven by the denitrifying bacteria. It is therefore an extremely important stage in the nitrogen cycle in the crust of planet Earth. There is a distinctive cycle in which nitrogen from the atmosphere is fixed, cycles through the biomass, is oxidized to nitrate by nitrification (see N-BART) and reduced back to nitrogen gas by denitrification which is controlled by the denitrifying bacteria.

The denitrifying bacteria are therefore an important indicator group for the decomposition of waste organic nitrogenous materials. These denitrifiers reduce nitrate through to nitrite and some continue the nitrification process on down to gaseous nitrogen (complete denitrification). In waters, the presence of an aggressive population of denitrifiers can be taken to indicate that there are significant amounts of nitrate in the water. Such waters are most likely anaerobic (free of oxygen) and relatively rich in organic matter. A common use for the presence of aggressive denitrifying bacteria in waters is that these bacteria signal the latter stages in the degradation of nitrogen-rich sewage and septic wastewater. Aggressive presence of denitrifiers 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. It is recommended that, where a high aggressivity is determined, the water should be subjected to further evaluation as a hygiene risk through a subsequent determination for the presence of coliform bacteria. In soils, the presence of an aggressive denitrifying bacterial population may be taken to indicate that the denitrification part of the soil nitrogen cycle is functional.

Denitrifying bacteria are not necessarily able to perform all four steps in the denitrification process and have been divided into four distinctive groups that can perform one or more of the various steps in the denitrification process. These are listed below:

Group 1 -step (1) only
Group 2 -steps (1), (2), and (3)
Group 3 -steps (2), (3), and (4)
Group 4 -steps (1), and (3) only.

Denitrification therefore serves as the major route by which complex nitrogenous compounds are returned to the atmosphere as nitrogen gas. There are four steps in the denitrification process:



One of the largest groups of denitrifying bacteria are the enteric bacteria which includes the coliform bacteria. All of these bacteria perform denitrification under anaerobic (oxygen free) conditions in a reductive environment. Some of the principal genera associated with denitrification are: *Actinomyces, Aeromonas, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Bacteroides, Campylobacter, Cellulomonas, Chromobacterium, Citrobacter, Clostridium, Enterobacter, Erwinia, Escherichia, Eubacterium, Flavobacterium, Geodermatophilus, Halobacterium, Halococcus, Hyphomicrobium, Klebsiella, Leptothrix, Micrococcus, Moraxella, Mycobacterium, Nocardia, Peptococcus, Photobacterium, Proteus, Pseudomonas, Rhizobium, Salmonella, Serratia, Shigella, Spirillum, Staphylococcus, Streptomyces, Thiobacillus,* and *Vibrio.*

As can be seen from the list, a very wide ranging number of bacteria are capable of denitrification. Their ability to perform denitrification is controlled, in part, by the availability of the nitrate, nitrite, nitrous or nitric oxide substrates.

The patented denitrifying bacterial activity reaction test biodetector (DN-BART™) has been designed to detect the aggressivity of the denitrifying bacteria that will reduce the nitrite to gaseous nitrogen (steps 2, 3 and 4). These bacteria are an important part of the nitrogen cycle in soils and waters. In waters, their aggressivity may be used to signal the fact that there is a significant degradation of nitrogenous material occurring.

Reaction Code, Denitrifying Bacteria

FO -Foam around Ball

Solution usually goes cloudy but the major positive for FO is the presence of very many bubbles collecting over >50% of the area under and around the BART ball to form a foam ring around the ball. This shows that complete denitrification has occurred and the denitrifying bacteria are present. There is only this single reaction recognized in the DN – BART that occurs when the nitrate has been completely denitrified to dinitrogen gas that collects as foam (interconnected gas bubbles) around the ball. This is more of a presence/absence test and the foaming usually is generated on the second test of testing at

room temperature.

Table One

The Relationship between Time Lag and the Population For Denitrifying Bacteria

Time lag (d)	12°C	212°C	28°C	36°C
1	314T	7M	9M	13M
2	88T	242T	159T	106T
3	23T	17T	8T	4T
4	6T	2T	790	330
5	1T	420	145	60
6	320	115	41	18
7	68	41	15	7
8	13	18	7	4

Note: populations are predicted active cells per ml (or p.a.c./ml) with M meaning millions and T meaning thousands. Note there are four temperatures presented. For more accurate populations predictions the use QuickPop that is downloadable from the web site: www.dbi.ca.

The denitrifying bacteria tend either to be aggressive and cause a rapid denitrification, or to be relatively placid. This test now functions through the detection of the complete denitrifiers. These bacteria reduce the nitrate to dinitrogen gas that appears as a foam ring around the ball. Generally, if the test is still negative after a time lag of two days, the population can be considered to be very small and non-aggressive.

Hygiene Risk – DN

Denitrifying bacteria flourish in waters that have sources of nitrate and organics. Such sources may involve wastewater that contain some septic material and could therefore present a potential hygiene risk. A coliform test should be considered to assess this risk where there is a detected population of denitrifiers (FO observed). Where the DN

population is greater than one thousand p.a.c. per ml, then consideration should be given to performing a coliform test routinely to determine the nature of any potential health risk.

Disposal methods

DN-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 field testers or 15 laboratory 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 field testers or 65 seconds for up to 15 laboratory 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.