# Biological Activity Reaction Test

# BART™

# User Manual

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Droycon Bioconcepts Inc.
315 Dewdney Avenue
Regina, Saskatchewan, Canada
S4N 0E7
Tel: (306) 585-1762
Fax: (306) 585-3000
Web: www.dbi.ca

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## 1.0 ABOUT THE *BART™*

The environment contains a myriad of different bacteria that are all capable of causing problems. These problems can range from slimes, plugging, discoloration and cloudiness to corrosion and infections. Such a wide variety of bacteria are not easy to detect and identify using a single test and yet their impact can make the water unsafe, unacceptable or unavailable due to losses in flow through plugging or equipment failure due to corrosion. The biological activity reaction test (BART™) is a water testing system for nuisance bacteria and can involve several different tests. These tests detect the activity (aggressivity) of these nuisance bacteria by the time lag (TL, measured in the number of days from the start of the test to when a reaction is observed). The longer the TL before the observation of activity, the less aggressive the bacteria are in that particular sample.

There are seven different tests that are recognizable by colored cap coding and the initial letters preceding the word BART™. These include selective tests for:

| | | |
|---|---|---|
| Iron Related Bacteria | IRB-BART™ | Red Cap |
| Sulfate Reducing Bacteria | SRB-BART™ | Black Cap |
| Heterotrophic Aerobic Bacteria | HAB-BART™ | Blue Cap |
| Slime Forming Bacteria | SLYM-BART™ | Green Cap |
| Denitrifying Bacteria | DN-BART™ | Grey Cap |
| Nitrifying Bacteria | N-BART™ | White Cap |
| Fluorescing Pseudomonads | FLOR-BART™ | Yellow Cap |
| Acid Producing Bacteria | APB-BART™ | Purple Cap |
| Biochemical Oxygen Demand | BOD-BART™ | Light Blue Cap |

Each of these bacterial groups cause different problems and often a combination of these tests should be used to determine which bacteria are present and causing problems. In the event that further information beyond presence/absence is needed, information on these reactions can be accessed using the Internet: www.DBI.ca. To read all of the reactions, lift the inner test vial carefully out of the outer BART™ test vial and view through the inner vial against an indirect light.

### 1.1 METHODOLOGIES

A common list of the methodologies and applications would be:

**IRB-BART™** test becomes positive when there foam is produced and/or a brown color develops as a ring or dirty solution. The TL (time lag) to that event is the delay**.** A negative has no brown color developing, no foaming or clouding. This test is commonly used to detect plugging, corrosion, cloudiness and color. The bacteria that may be detected by this test include iron oxidizing and reducing bacteria, the sheathed iron bacteria, *Gallionella*, pseudomonad and enteric bacteria.

**SRB-BART™** A very simple test to perform in which a positive test occurs when there is a blackening either in the base cone of the inner test vial (80% of the time) or around the ball (20% of the time). The culture medium is specific for the sulfate reducing bacteria, such as *Desulfovibrio* and *Desulfotomaculum*. This is a more specific test and specifically relates to corrosion problems, taste & odor problems ("rotten egg" odors), and blackened waters. Slimes rich in SRB tend to also be black in color. A negative indication occurs when there is an absence of blackening in the base cone of the inner test vial or around the ball.

**HAB-BART™** There is a very real need to determine the amount of heterotrophic aerobic bacterial activities in some wastewater, particularly those that are aerobic. Here, biodegradation may be a primary concern, such as on a hazardous waste site. This test relies upon the ability of the heterotrophic aerobic bacteria to reduce a methylene blue dye. To add the methylene blue to the sample, the test vial once charged is simply placed upside down for 30 seconds or 5 minutes in a saline environment, to allow the blue color to develop. A positive is detected by the blue color becoming bleached (due to the activity of methylene blue reductase). Bleaching may begin at the base of the test vial or just below the ball. Note that a residual blue ring is likely to remain around the ball, but this does not mean heterotrophs are absent. A negative indication occurs when there is an absence of the blue color becoming bleached. This test is used to detect slimes, plugging, taste & odor, cloudiness and can also detect the amount of aerobic heterotrophic activity on hazardous waste sites.

**SLYM-BART™,** some bacteria can produce copious amounts of slime that can contribute to plugging, loss in efficiency of heat exchangers, clouding, taste and odor problems. This is one of the most sensitive BART™ tests. A positive involves a cloudy reaction in the inner test vial often with thick gel-like rings around the ball. A negative test remains clear.

**FLOR-BART™** A major group of aerobic heterotrophs are the pseudomonads. These bacteria are very well adapted to breaking down some chemicals such as jet fuel and solvents but also can infest recreational waters and cause conditions ranging from skin, eye, ear, and nose infections to pneumonia-like infections. The infectious pseudomonads do produce an ultra-violet fluorescence that is usually a pale blue color. Presence for this test means that either a greenish-yellow or a pale blue glow is generated by the careful application of an ultraviolet light just below the ball. The degraders tend to generate the greenish-yellow glow while the health risk group generates the pale blue glow. A negative indication occurs when the sample remains clear.

**DN-BART™** Nitrates in water are a serious health concern particularly for babies. There is one group of bacteria called the denitrifying bacteria and many of these are able to reduce the nitrate to nitrogen gas. In this test, this gas forms a foam of bubbles around the ball, usually within three days. The presence of this foam by the end of day two 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. This test is applicable to any waters where there is likely to be potential septic or organic contamination. The presence of denitrifiers would indicate a potential health risk due to either septic wastes or nitrates in the water.

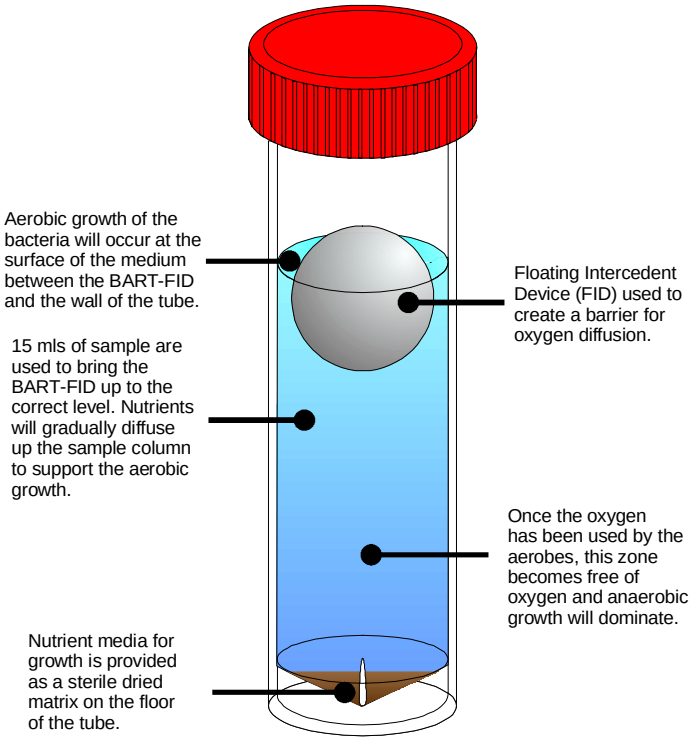
## 1.2 The six W's of the BART™ Testers

There are numerous ways in which microbes can become a nuisance in water. Often these events are ignored, considered inevitable or put down to simple physical and chemical effects. Ignorance may be bliss, but it is expensive. These microbes can cause corrosion, plugging, failing water quality and the shortening in the life span of the installation. In today's world, disposability is being replaced with sustainability and ignorance replaced with knowledge. There has been a considerable lack of attention paid to the nuisance events caused by bacteria other than those associated with hygiene risks. In ground waters, it has been a common practice until a decade ago to consider the environment to be essentially sterile and so microbial events were not considered important. In surface waters, larger and more obvious organisms tended to receive more attention than the slimes and clouds in the water. Today, it is becoming recognized that microbes are present in all waters and that they have a nuisance impact that needs to be managed if sustainability is to be achieved. This document addresses the advantages of becoming more aware of the microbes and their activity in water. It should be remembered that there is no such place as a totally sterile water environment and that, if the microbes are active, there will be affects on the environment.

### 1.2.1 WHAT ARE THE BART™ TESTERS?

BART™ stands for the patented biological activity reaction test. As the name implies, the test detects biological activity by looking for activities and reactions. Activities relate to growth events such as the formation of clouds, slimes, and gels. Reactions relate to the manners in which the microbes interact within the BART™ test. These reactions may take the form of color changes, generation of gasses, and precipitation. The unique nature of the BART™ test which makes it very different, and possibly superior, to the agar techniques is the fact that the water used in the test all comes from the sample and contains the microbes still within their natural environment. The water in the agar methods comes with the agar but it is tightly bound. This means that the microbes have to be taken from the water, placed into contact with the agar surfaces, and expected to "mine" the bound water for growth from the agar. Many microbes in the environment are not able to easily do this and so may be missed using agar cultural techniques (i.e., no grow, no show, no count and so not important).

The BART™ uses a unique system for encouraging the microbes to grow in the test. First, there is normally no dilution of the sample. Secondly, the sample becomes adjusted to a variety of different habitats by the nature of the BART™. Thirdly, the microbes that can be active and/or react with the selective conditions created within the BART™ test can be considered to belong to a specific group of bacteria (e.g., iron related bacteria). These selective conditions are created using two devices. The first (1) is a floating ball, FID-floating intercedent device, that restricts the entry of oxygen into the sample below. The second device (2) is the use of a crystallized deposit of selective nutrients, which sits in the bottom of the tube and encourages the activities and reactions by a specific group of microbes. In the first device, the oxygen enters around the floating ball to allow oxygen requiring (aerobic) microbes to grow. They will use all of the oxygen diffusing down so that the sample further down becomes devoid of oxygen. This volume underneath becomes suitable for the growth of microbes that do not require oxygen (anaerobic). Thus, the single BART™ provides environments which are aerobic (oxidative) and anaerobic (reductive). Essentially this is a reduction-oxidation gradient with a transitional zone (redox front) in the middle.




Sergei Winogradsky originally reported this type of phenomenon forming in waters kept in tall glass cylinders at the end of the nineteenth century. The beauty of this device is that different microbes prefer to function at different sites on the redox gradient and so can be seen being active and reacting within that zone. Very often, the first sign of this is the development of a cloud of growth that may be fuzzy and diffuse or a flat plate floating in the watery medium.

The key to determining the presence of different groups of microbes is the crystallized selective medium attached to the floor of the BART™ device. This medium will begin to slowly dissolve when the sample is added. As the medium dissolves, a series of chemical diffusion fronts become established and move slowly up the BART™ tube. This slows upwards progression which can take as long as two days, gives the microbes in the sample time to adapt to the increasing concentration of nutrients and, if suitable, begin to become active. Even the very sensitive microbes that would normally fail to grow on any agar media are better able to adapt and grow within a BART™ test if the crystallized medium is suitable for their growth. The location of the growth gives an early indication of the type of microbes involved. Activity in the base of the BART™ test would tend to suggest anaerobic organisms while activity at the top around the ball is more likely to be aerobic. Often the activity may center along the diffusion front for the dissolving crystallized medium. When this happens, the microbes are likely to be able to grow under aerobic and anaerobic conditions (facultative anaerobes).

Given that the BART™ test presents a whole range of environments for the microbes to grow, the key becomes the form of the crystallized culture medium that is in the BART™. It is this factor that causes different communities of microbes to become active and, hence, be detected. The eight BARTs™ employ different culture media to make the test selective. These are listed below (Table One) defining the microbial group first (acronym is given in brackets) followed by the form of the selective culture medium used.

**Table One**

**Principal Microbial Groups Determinable Using the BART™ Biodetectors**

| Microbial Community | | Selective Culture Medium* |
|---|---|---|
| Iron Related Bacteria | **IRB** | Winogradsky's medium |
| Sulfate Reducing Bacteria | **SRB** | Postgate's medium |
| Slime Forming Bacteria | **SLYM** | Glucose Peptone medium |
| Heterotrophic Aerobic Bacteria | **HAB** | Sugar Peptone medium |
| Algae | **ALGE** | Bold's medium |
| Fluorescent Pseudomonads | **FLOR** | Peptone base medium |
| Denitrifying Bacteria | **DN** | Nitrate Peptone medium |
| Nitrifying Bacteria | **N** | Ammonium salts medium |
| Acid Producing Bacteria | **APB** | Modified Glucose Peptone medium |
| Biochemical Oxygen Demand | **BOD** | Modified Sugar Peptone medium |

* Media have been modified to maximize the potential for recovery of the microbes using the BART™ system.

Considerable attention has been paid to drying these media in a manner so that they do not loose their selective function and are not able to re-hydrate until the sample is added. Each medium is dried in a different manner to ensure a stabilized form. To prevent re-hydration, the test vials are immediately packed in foil pouches, which act as effective moisture barriers. A three-year shelf life can be achieved when stored in a cool, dry place. Refrigeration is not necessary.

## Reading the Results Using a BART™ Testers

There are two important forms in which information can be obtained. These relate to the type of consortial (community) microbial activity that may be occurring and the determination of the population. The consortial microbial activity can be determined from the reaction patterns observed. Essentially, the reactions displayed can be used to build up a "picture" of the community (consortium) of microorganisms in the sample being tested.

Populations are determined by the length of the time lag with the proposition that the longer the time lag to the detection of a reaction, the smaller the aggressive population of the microbial consortium being determined. This time lag is normally measured in days to the first detection of a reaction. Since the BART™ tests each detect a different consortial population, the time lag for one consortium does not directly relate to the time lag for a different test type.

### Interpreting the Test Data

It is relatively easy to interpret a negative test because the bacterial groups do not generate any signals of activity in the BART™ test. A positive detection means that: (1) a minimum number of bacteria must have been present to cause the observed activity and reaction, and (2) that the TL to that observation can be used to project the population size. The following tests are normally used at the presence-absence level:

**N-BART™** minimal population detected: 1,000 cfu/ml
**FLOR-BART™** minimal population detected: 100 cfu/ml

It should be noted that lower detection limits can be achieved by extending the TL for the N-BART™ before examining the contents for the presence of nitrite, a transitional bi-product of nitrification.

The remaining tests can either be used as a presence/absence or as a semi-quantitative test by determining the TL at which the first positive activity/reaction occurred.

For determination of more acurate populations and/or populations at $28^0$C, Quick Pop software can be used. The sowtware can be downloaded from www.DBI.ca.

### Recognizing Positive Reaction Patterns

Reaction Patterns are the visible evidence that some activity is occurring in the BART™ tester as a result of the interaction between the active microorganisms in the sample with the redox gradient and the chemicals diffusing upwards from the base of the inner BART™ test vial. As a result, reactions range from clouded zones and gas bubbles to changes in the color of a part or the whole length of the sample. Each reaction pattern will be addressed by BART™ type.

## 1.2.2 WHY USE BART™ TESTERS?

The BART™ testers have two major advantages:

1. You do not need a laboratory to set up the test to determine whether there are aggressive bacterial problems in the sample being tested. They are easy to read since the signals (reactions) generated are observable.
2. The BART™ testers provided a greater variety of environments within which the bacteria of concern can grow. This is a very major advantage over the traditional agar techniques in common use in the microbiology laboratories today. This feature makes the BART™ testers far more sensitive and reactive to aggressive bacterial populations.

These are two main advantages in using the BART™ testers since they offer convenience, simplicity, sensitivity and durability.

Convenience means that the techniques employed to set up the test is easy to follow. The BART™ testers are supplied in individual, moisture proof, foil pouches that prevent the tester from degenerating as a result of premature rehydration. Within the foil pack is the double tube tester. The outer tube acts as a:

- Protection from damage to the inner test vial in which the test is actually performed.
- Security measure to reduce the risk of any odors and accidental leakage generated from the inner test vial escaping from the tester.
- Providing a bigger base for the tester so that it is more stable and less likely to be knocked over.
- Convenient determination of any reactions without having to directly handle the inner test vial after the test has been started.

Meanwhile, the inner tube offers all of the patented advantages of providing a very broad spectrum of environments in a watery environment where the different parts of an aggressive bacterial consortium can elect to grow. These environments can be described in broad terms as changing primarily with the descent down beyond the ball:

- There is a water film covering the top 20% of the surface area of the ball above the surface level of the sample. Biofilms can grow up into this very oxidative zone to be seen as a slime-

like coating. Slime forming bacteria and molds are two common groups of microbes that can grow at this location.

- The sample above the equatorial region of the floating ball. Here, the liquid medium remains saturated while oxygen diffuses downwards from the atmosphere above the floating ball. As a result, it is common for the aerobic slime forming bacteria to grow into various types of slime-rich ring-like structures. Additionally, these biofilms that form slime rings entrap any gas bubbles being generated deeper down in the active inner test vial. These gas bubbles bounce up around the lower side of the ball and get caught up in the mass of biofilm growth to cause a foam that collects around the ball. Molds (fungi) will also grow in a commonly fuzzy manner down into this zone. This site is very oxidative and the chemicals may be moved into colored oxidized states (e.g., reds, browns and yellows).
- In the liquid medium just below the ball (usually between 3 and 8 mm below) there is a reduction – oxidation (redox) front where the oxidation-reduction potential (ORP) changes from a positive oxidative state above to a negative reductive state below. Many aggressive bacteria tend to grow first at this redox front. Normally, this will take the form of cloudy growth that may be very "fuzzy" in form or quite "tight" and form very distinct plate-like structures in the medium. Bacteria growing at these sites are commonly a mixture of aerobic and facultatively anaerobic forms able to utilize, directly or indirectly, the selective medium diffusing up the liquid medium column in the inner test vial.
- Below the redox front in the lower third to half of the liquid medium, the ORP is negative (reductive). This means that only those bacteria able to grow anaerobically (without oxygen) will be active here. Commonly, there is more color reactions at these sites associated with the reductive end products (e.g., blacks and greens). Generally, visible growths are more gel-like (colloidal) and denser.
- Inside the base of the inner test vial, two major events occur. First, the medium crystallized into the floor dissolves and diffuses upwards meaning that the deposited chemicals disappear and, commonly in some of the BART™ testers, it is possible to see the liquid medium through the base. Second, there are reactions within the inner test vial that cause changes in the color and texture of the basal chemical deposits. These reactions can cause the base to blacken or change to a different color. It should be noted that the occurrence of a white deposit commonly occurs in an IRB-BART™ but has not yet been assigned as a significant reaction.

The BART™ has two modes in which it can be used. For the field testing where the BART™ tests are actually performed in the field, then the full BART™ test should be used in which the outer tube gives the additional advantages discussed above. In the laboratory setting, the outer tube is redundant since the inner test vials are being used in a more secure environment. As a result, well-equipped laboratories with trained technical staff may prefer to use the more economical LAB-BART™ versions of the standard field test. This test (LAB) is packed in units of fifteen tests rather than the standard BART™ tester (with outer tubes) that are packed in units of nine tests.

### 1.2.3 WHO SHOULD USE THE BART™ TESTERS?

Gradually, the roles of bacteria in the myriad of natural and engineered events are becoming appreciated. These range from the obvious (e.g., taste, odor, corrosion and slime formation) to the subtle (e.g., bioaccumulation and occlusion). Virtually any management practice involving water could be subjected to the impacts of bacteria and other microorganisms and the BART™ testers

provide a means to monitor either the state of the microbial aggressivity or the impact of a treatment.

For managers of water systems, there is a need to understand the potential and real challenges that can be caused by these nuisance microorganisms. Unfortunately, very often, microbiological fouling of a system (whether the base medium is water, oil or gas) is slow and covert without any obvious signals to show that it is microbial in origin. Often, these degenerative processes are put down to the normal aging of the facility and it is not considered that these processes could be driven by microbes and managed by monitoring the levels of aggressivity in these nuisance bacterial events using the BART™ testers.

Who should use the BART™ testers? Anyone who understands that bacteria and other microbes can affect the lifespan of a facility in a very real manner. These effects can range through a whole range of characteristic changes including:

- Corrosion in which the microorganisms corrode the solid structures (e.g., steel or concrete) in such a manner as to severely weaken the structure causing failure.
- Plugging in which the microorganisms form thick biofilm growths (slimes) within porous media which causes significant losses in conductivity (hydraulic or thermal).
- Radical changes in water quality caused by the casual sloughing of the slimes which are loaded with microbial cells and their associated accumulates. This sloughing can cause sudden dramatic changes in the concentrations of some chemicals (e.g. iron and phosphorus) in the water.
- False data generation due to the biofilms within the upstream zone above the site of special interest. These biofilms (or slimes) can accumulate vary large concentrations of recalcitrant chemicals that would otherwise have found their way into the sampling site. This is a form of bio-filtration and accumulation which gives a falsely improved water quality until the growths begin to slough. Monitoring wells may be particularly prone to these events when organic pollutants (e.g., BTEX, PAH, VOH) approach the well and are accumulated into the biofouled zone around the well. This biological interface acts as an effective filter until maturation causes the collapse of the biofilm structures.
- Odors can be generated by a whole range of microorganisms with some of the most well known being:

    (1) rotten egg (SRB generating hydrogen sulfide),
    (2) fishy (commonly heterotrophic aerobic bacteria and, in particular, *Pseudomonas* species),
    (3) earthy-musty (geosmins generated primarily by the *Streptomyces*),
    (4) septic (generated by various members of the enteric bacteria including the coliform bacteria) and
    (5) vegetable/fruity odors (from a variety of algae and yeast).

    One useful tool to aid in the confirmation of the source of odors is that the odors will concentrate between the outer tube and the inner test vial of the BART™ test when odor-generating microbes have grown in the tester. Loosening the outer cap and cautiously "sniffing" the gap between the cap and the outer tube will reveal the types of odors being generated by these microbes. Often this smell is coincident with an odor being detected in the sample itself. This can often convince a doubter that it is the microbes in the BART™ that are capable of causing the odor problem and a focus on managing the problem is now understood.

- Turbidity has often been thought of as simply a chemical event associated with chemical colloids, silts or precipitation. These will cause the sample to go cloudy. More commonly than not, the cloudiness in the sample is a combination of turbulence swirling up sediments into the liquid medium and the growth of microbes within that sample. If the cloudiness is microbial, then it can be expected that the BART™ testers will detect very aggressive microbial populations.
- Color is most commonly generated by microbes through the accumulation of iron (yellows, browns, reds and oranges) although occasionally pigment can be generated by the microbes themselves as pigments. These pigments are most commonly browns, yellows, greens, blue-greens and reds and are generally more transient.
- Biodegradation is a major industry today as a part of the environment industry. Where there is a biologically driven degradation occurring, there is an inevitable increase in the aggressivity of those microbes in the environment that are associated with an observed degradation. To monitor this aggressivity, the BART™ testers can be used. Generally, if the degradation is basically aerobic and involves a narrow spectrum of organic pollutants, then the heterotrophic aerobic (HAB), the fluorescing Pseudomonad (FLOR) and the slime forming (SLYM) BART™ testers are most likely to detect the increased aggressivity of the degraders. This can then be used as a "benchmark" for the vitality of the microbial consortium causing the degradation. If the degradation is anaerobic, then a different spectrum of bacteria may be the most aggressive. These could include the sulfate reducing (SRB), the slime forming (SLYM) and the denitrifying (DN) bacteria.

The BART™ testers are suitable as a field test for any manager or consultant concerned about managing problems which are likely to be either instigated by, or worsened by, the presence of the various groups of microorganisms detectable using the BART™ testers. Just who would use the BART™ testers would depend upon the level of biological activity occurring whether this be biofouling, biofiltration, or biodegradation. Some examples of who would use the BART™ testers are listed below:

- **Water Well Operators**. Water wells are a "site unseen" operation. The extent of any visible fouling is limited to camera logs down the well or obvious fouling of filters and lines downstream of the well head. Often, the bulk source of all of the biological activity is outside of the well screen and not visible. What is visible is the "tip of the iceberg" which is the colloidal structures floating in the well water column (well snow), encrustations, tubercles and slimes attached to the walls and screens of the well and as deposits in the bottom of the well. Detecting even the most aggressive bacteria under these conditions is not simple. The bacteria often have to be "tricked" by changing the normal operational procedures for the well in order to be able get them into the water so that they can be detected using the BART™ testers. Most commonly used of the BART™ testers are:
    - the IRB (where there are known iron problems);
    - SRB (where there are anaerobic, black water and corrosion problems);
    - SLYM (where there are slimes forming in and over the well casing, screen or pump); and
    - HAB (if there is turbidity, odd odors, cloudiness, fluorescence and high organic loadings in the water).
- **Water Treatment Plant Operators**. Water treatment facilities usually involve water that has become aerated, possibly filtered, disinfected, clarified and stored. It should be remembered that the BART™ testers are proofed against the possible effects of chlorine based disinfectants by the inclusion of a neutralizer that is effective for concentrations of up to

5,000 ppm of chlorine. In general, apart from the concern for the elimination of coliform bacteria from the water (see the separate section on the COLI-BART™), there is little regulated limitations to the microbial loadings in potable, industrial and recreational waters. Consequently, the need to monitor nuisance microbes is more in the interest of the operator rather than regulatory compliance. Unfortunately, the common attitude that water should be free disenfranchises the ability of the operator to assure a maximum operational efficiency in favor of bulk acceptable water produced at the lowest cost. Biofouling causes many covert (and commonly negative) impacts which often go unnoticed until it is too late to effectively control and then radical "surgery" has to be performed to replace the fouled parts. Common problems relate to massive slime formations (SLYM and HAB are good for checking this), corrosion of equipment (SRB), encrustations in pipes, tanks and filters (IRB and FLOR), and sudden fluctuations in water quality (HAB, SRB and DN). Fluctuating nitrate problems could be related to changes in the biofouling with a greater probability of nitrate expression in waters high in oxygen and low in organics. The organics would trigger a greater rate of denitrification particular under a suppressed oxygen regime. Routine use of BART™ tests in the ongoing operations of the treatment plant can allow earlier control of potential serious biofouling events.

- **Bottled Water Plant Operators**. Bottled water represent a growing fraction of the consumed water since it reflects a superior product in the minds of the consumer to potable water supplies provided by local agencies. While ozonated and carbonated waters do have the microbial loadings suppressed to varying degrees depending upon the techniques employed, there is still a potential for the water to degenerate as a result of microbiological activity. Most commonly, this will take the form of clouding, deposits, tastes and odors. If these events occur when the product is already with the distributor or final retailer, then this would have serious consequences for the bottling company. Quality assurance and quality control can be achieved using the BART™ testers to determine that the source water is not fouled with aggressive bacteria and that the ozonation or carbonation has effectively acted as a disinfectant to suppress the nuisance microbes.
- **Environmental Managers**. The largest biomass by far on Earth belongs to the microorganisms. This group is not sitting there passively while the biota (animals and plants) quietly does all of the "work". Microorganisms are ubiquitous and functionally active whether they are in the human body (90% of the cells in the human body are microbial cells), in soils, waters, oil and gas, muds and sedimentary rocks. Environmental managers face the task of "managing" the environment and it is essential that the role of microorganisms in that environment be recognized. The BART™ testers offer the potential to take "snapshots" of the aggressivity of the various components in the microbial biomass that can have a significant impact on the environment of concern.
- **Sanitary Landfill Operators**. There are a number of microbial challenges faced by sanitary landfill operators simply because of the highly organic nature of the fill materials deposited in the landfill. In going down through a landfill, there are a series of stratified activities predominantly microbial in form. These include (going from the top down):

    - Surface growths on the redox front dominated by methanogenic bacteria that are able to degrade methane.
    - Biogas generation zone in which methanogenic bacteria are very active producing copious quantities of methane.
    - Drainage systems in which bacterial activity causes the generation of thick plugging slimes (dominated often by SRB and SLYM bacteria). Should these growths get too

aggressive, then there could be reduced permeability that would lead to the water mounding in the landfill and breaking out through side erosions.

- Leachate outflows from the drains. Very aggressive aerobic activity is likely to occur around the redox fronts at these sites leading to radical nitrification (nitrate production) and heavy slime growths (dominated by HAB, SLYM, FLOR and IRB).

Both the functionality and stability of sanitary landfill operations can be severely compromised by aggressive microbial activities. An ongoing monitoring of these nuisance microbial groups using the BART™ testers can aid in predicting and controlling problems before they become uncontrollable.

- **Operators of Recreational Waters**. These waters range from spas, swimming pools, hot tubs and beaches. With these waters there is a primary concern to reduce the hygiene risks to the users by the routine examination for coliform bacteria. However, there are other problems particularly with hot tubs, swimming pools and spas that are caused by other nuisance bacteria that can be detected using the BART™ testers. The effects of the nuisance bacteria would fall under the categories of reducing plant efficiencies, reducing water quality, and generating unacceptable slime growths. There are both economic and user acceptability issues involved in the microbial biofouling problems which can be monitored and managed using the BART™ testers.
- **Irrigation Operators**. Vast volumes of water are used in the irrigation industry. This water is subjected to radical changes in pressures and flow rates often under increasingly oxidative conditions. Such shifts in conditions can cause a focusing of microbial slime growths within the system and nozzles that can radically reduce efficiencies and increase operating costs. Most commonly, the SLYM and IRB are likely to dominate under low iron and high iron conditions respectively. If there is a low oxygen concentration in the water, high sulfates or hydrogen sulfide ("rotten" egg odor, black water), then the SRB may be dominant in the irrigation system. Cleanliness and sanitization of the equipment (confirmed by the routine use of the BART™ testers) is likely to pay dividends through improved efficiencies and higher quality water for irrigation.
- **Hazardous Waste Site Operators**. While these sites may be very hazardous to humans, the environments created may be very conducive to extensive microbial activity. Such activity can be related to the rates of biodegradation and bioaccumulation activities being generated by the naturally attenuated consortia active at the site. Additionally, the operation of treatment facilities, injection and recovery wells, distribution lines and storage tanks can all become severely compromised. For example, injection wells returning treated water back into the formation has often become aerated (oxidative) and, upon injection, forms a redox front around which bacterial slime growths would form causing erratic reductions in permeability. For the operator of hazardous waste sites, the BART™ testers provide a simple monitoring tool to determine the level of bacterial activity occurring when used routinely. Management of the site can subsequently be improved through this routine monitoring of the levels of aggressivity (most simply monitored by the time lags observed).
- **Cooling Tower and Heat Exchanger Managers**. As a matter of routine, water is used as the heat sink in many processes. The heat in that water is removed to the air (e.g., cooling tower) or to a greater volume of water (e.g., heat exchanger). For the heat to move efficiently in the transfer from the water to the receiving medium there should be no interferences. Biofilms (slimes) forming at these interfaces can severely reduce this heat exchange in several ways. Failure to control these biofilms can be expensive due to losses in the process efficiency that causes equipment to fail to meet specifications. Controlling the biofilms is usually achieved by the application of biocides. By the routine testing of the waters using the BART™ testers, the effectiveness of the biocides in suppressing the biofilms can be determined conveniently

and easily. Increases in the aggressivity can be determined by the shortening in the time lags while the success of a biocide treatment may be seen through lengthening of the time lags. As a rule of thumb, a one-day increase in the time lag reflects a one order of magnitude reduction in the numbers of bacteria in the water.

### 1.2.4 WHERE TO USE BART™ TESTERS?

BART™ testers were primarily developed to determine the aggressivity of different groups of bacteria in water. The reason the BART™ testers are so suitable for the determination of the types of aggressive bacteria is that so many different environments are presented in such a small volume (15ml). When there is activity, this is recognized by activity within the test vial that may be seen as such events as color shifts, cloudiness, and gassing. These are convenient to observe and so a full laboratory is not necessary in order to conduct BART™ tests. As a result, the tests themselves can be performed away from the laboratory in an office, a field station, even in a trailer, a tent or even in a hotel room! It should be remembered that the BART™ format for field use has the outer tube that provides an additional barrier to prevent possible odors or leakage coming out from the inner test vial.

One important question is always "What temperature should I use to keep the BART™ test at while they are running?" Microbiologists usually refer to the temperature at which the BART™ test is "running" as the incubation temperature. Commonly, the incubation temperature of choice is room temperature and that can be anywhere from 19$^{o}$C to 25$^{o}$C. Samples can range in temperatures from 4$^{o}$C to 35$^{o}$C. The ideal would be to operate the BART™ tests within 5$^{o}$C of the temperature at which the sample was taken. For waters with temperatures of between 15 and 35$^{o}$C, room temperature may be fine since the maximum difference between sample and incubated temperature would be 17$^{o}$C and, commonly, it would be less than 5$^{o}$C. If the water was sampled from a site where the temperature was less than 15$^{o}$C, then the types of bacteria that would be aggressive would probably grow better at lower temperatures. These types of bacteria are called "psychrotrophic" and can probably best be grown in a refrigerator set at 8 to 10$^{o}$C. This is not a very cold setting but would be within the optimal growth range for many psychrotrophic bacteria. In tropical countries where the temperature is close to blood heat year round, then optimal incubation conditions would at 35 to 37$^{o}$C. This could be undertaken in a room that is not air-conditioned. It should be remembered that the BART™ tests should always be incubated out of direct sunlight although regular room lighting does not appear to affect the tests. The exception to this rule is the ALGE-BART™ that does require indirect sunlight or continuous daylight fluorescent lights to allow the algae to photosynthesize.

Where water samples have been obtained from source water at temperatures higher than 35$^{o}$C, there is a concern about where and how to conduct the BART™ tests. As a rule of thumb, the incubation temperature should be, in these circumstances, within 10$^{o}$C of the original water temperature (preferably within 5$^{o}$C above that temperature). To conduct these tests, there would need to be an incubator adjustable to those temperatures or a very warm location would have to be found. It should be remembered that the safe upper limit for incubating the standard BART™ test is 70$^{o}$C. Above that temperature, the grade of polystyrenes used in the test vials begins to lose its structural integrity and buckle.

### 1.2.5 WHEN TO USE THE BART™ TESTERS

There are three conditions under which the BART™ tests may be used:

1) To determine the cause of abnormal event that may involve microbial activity

2) To monitor the effectiveness of a treatment designed to control the abnormal event diagnosed as being at least in part microbial
3) To effectively prevent a recurrence of the abnormal event through an ongoing testing and reactive treatment scenario.

Each of these three circumstances involves a different approach. For condition 1, the type of BART™ to be used is not certain because an abnormal event has occurred that is thought to involve microbial activity. As a result of this uncertainty, a broad spectrum of BART™ testers should be used to test the water sample. Commonly, the range of testers that could be used would include (aerobic conditions):

HAB, SLYM, IRB, FLOR and SRB

While under anaerobic conditions, a different spectrum of BART™ testers may be selected:

SRB, BIOGAS, SLYM and IRB

For condition 1, the sample should be taken from the site where the abnormal event is occurring or just downstream of the event. Remember that for most of the time greater than 90% of the microbes are in slimes (biofilms) attached to surfaces and so these would not even be present in the water sample! A negative BART™ does not mean a negative problem but simply means that the bacteria causing the problem were not in the water sample being tested (they were in the slimes the water passed over before being sampled).

For condition 2, the circumstances are slightly different in that BART™ tests have already been conducted and, normally, the time lags would be different, at least marginally, for each of the types of the BART™ tests used. Commonly, it is the two BARTs™, which have the shortest time lag that may be selected to determine whether a treatment management strategy now being applied to the sample is effective. It should be remembered that BART™ tests giving longer time lags might also be important. This is particularly true of the IRB-BART™ that can produce complex reaction patterns that reflect the form of the bacterial consortium in the sample (seen as the sequence within which the reactions actually are observed). If these reaction pattern signatures do shift during the treatment, then there is a list of the meaning of each reaction pattern signature (the order in which the reactions occur) in terms of which type of bacteria are dominant.

Essentially in condition 2, the objective is to try and evaluate the success of the treatment strategy applied primarily through the impact on the time lags. A dogmatic interpretation of this would be that:

- For each day of additional time lag delay, it can be considered that there would be one order of magnitude reduction in the population for each day's lengthening in the time lag. For example, a water sample contained 100,000 cfu/ml and had a time lag of 2 days before treatment and this lengthened for 4 days after treatment. This meant that the treatment cased a two-day lengthening to the time that would be two orders of magnitude (99% reduction to 1,000cfu/ml).
- If the time lag did not increase or decrease, then the treatment applied did not have any effect on the aggressivity of the bacteria being monitored using the BART™ tests.
- If the time lag shortened after the treatment, then not only did the treatment prove to be ineffective, but it created a condition in which the bacteria were able to become more aggressive. This stimulation of the bacteria may have been due to either: (1) the treatment including chemicals that could directly stimulate the bacteria (e.g., ineffective organic biocides, phosphates, organic carriers); or (2) the treatment could cause the release of bacteria into the water that had been attached. Remember that, in the latter case, when the

bacteria are in the attached state, they will not be in the water and so essentially could be missed as simply not detected. In this case, the treatment may have worked effectively at dislodging the attached (sessile) bacterial growths, but had not killed, or removed, the cells from the water being tested.

Condition 2 is one that would be used to begin to determine whether the treatment was effective at controlling the microbial driven problem and also which BART™ testers could most conveniently be used to determine the effectiveness of an elected treatment management strategy.

Nothing lasts forever and so a single treatment of a water problem should not be viewed as ending the problem forever! Condition 3 is an essential part of a preventative maintenance strategy. Here, one or two types of BART™ testers are used in a routine manner to check to see that the water is still showing the lower bacterial activity level that was achieved by the treatment. If the time lags return to the pre-treatment levels then, clearly, the treatment may need to be repeated to again suppress the bacterial activity. If the time lag begins to shorten, then there is that potential to conduct a lower intensity of treatment to return to time lag to the post-treatment lengths. For water wells, one common scenario is to conduct monthly testing with just one or two BART™ tests (e.g., IRB and SRB). If the post-treatment time lags for these were 10 and 14 days respectively, monthly testing would show that the recovery was holding if the time lags remained the same. In practice, it may be determined that a time lag of 8 or 10 days respectively was a concern. It may be that the water sample contained some sloughing material that triggered the shorter time lag. Repeating the testing but this time does confirmation with duplicate BART™ tests to determine whether the aggressivity was a result of a chance sloughing or the bacteria becoming more aggressive. If the time lags remain shorter in that duplicates, then a preventative treatment would need to be applied to again suppress the bacteria and get the time lags back to the longer (and more acceptable) levels.

It is not responsible to propose the same time lags as being acceptable for water systems of all the various types that require management. Since each water system or well offers some unique parameters, it is much better for the routine (conditions 1, 2 and 3) be followed and a practical strategy developed that is appropriate to that water. The target should be set in the light of the activity associated with conditions 1 and 2 and then used to support sustainable water management of that system with a minimum of diversions from the established schedule of testing and treatments. Condition 3 would then be used to operate the system with the confidence of an “advanced warning system” and a treatment that has been validated by repeated appropriate application. Should the treatment start to fail, this may be because the microbes causing the problem have adapted to that treatment. It should be remembered that microorganisms are adaptable and they have the ability to adapt to treatments when these are used repeatedly. The history of antibiotic therapy is plagued with failures due to the adaptation of the targeted bacteria to the treatments.

### 1.2.6 WHICH BART™ TESTERS TO USE?

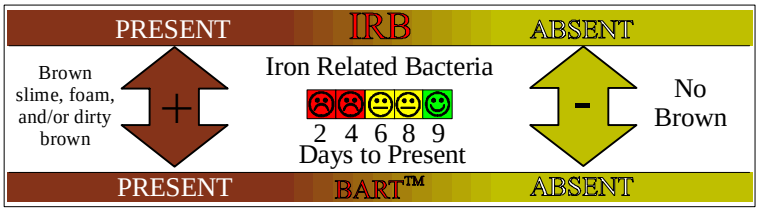
Some would call this the million-dollar question! There are two ways to address this question:

(1) Define the environments that each of the BART™ testers can best be used to detect microbial aggressivity
(2) Take each environment and define which BART™ testers would be most appropriately applied on condition 1 events.

This assumes that many of the applications of the BART™ testers would begin with an imminent or serious problem for which rehabilitation is urgently required. It has to be

remembered that bacterial consortia may not be detectable by just one of the BART™ testers. Sometimes the consortium can cause reactions in more than one of the BART™ testers. This means that there can be over-lap. The range of detection of nuisance microorganisms will be discussed below for each of the major BART™ testers.

## 2.0 IRON RELATED BACTERIA, IRB-BART™




Iron is well known to be a critical substance for all life. In animals, it is a common part of the mechanisms for moving oxygen throughout the living body. Because iron plays such an important function in the energy metabolism, there is considerable biological competition for iron. Microorganisms also compete for iron and the use of various types of proteins called siderophores (e.g., hydroxamates and catechols). Additionally, many bacteria can also bind ferric ($Fe^{+++}$) ions into chelating structures know as ligands. This means that many bacteria are able to bind and hold iron in many forms to make large iron-rich structures that are sometimes seen as encrustations, tubercles and bog iron ore deposits. Little is known of the possible use of this iron to generate electro-motive forces (EMF) as a part of the growth of these iron-related bacteria. There is one group of bacteria, called the magnetotactic bacteria, which actually posses small magnet-like structures (magnetosomes) and are able to sense magnetic fields.

So complex are these various biochemical systems for holding onto iron, the precise nature of these events remain only partially understood. However, there are many bacteria which can continue to accumulate iron to the point that the growth becomes almost saturated with oxidized iron and forms a hardening clog or encrustation. Such mineralizing growths may also incorporate carbonates and sulfides with a high iron content (going from 1% up to as high as 40% dry weight) and reducing organic content (declining to as low as <1% organic carbon). The formation of hardening clogs/encrustations can seriously impair the designed hydraulic characteristics of the infested region, causing degenerated water quality and production capacities.

In using the IRB-BART™ to examine waters for the presence of iron related bacteria, it has to be remembered that iron bacteria grow predominantly on surfaces and not directly in the water. When testing water, the BART™ user has to assume that the IRB have detached, are suspended, and possibly are active in the water. As a consequence of this problem, there is a potential for an IRB-BART™ to give a “false” negative since the IRB are absent from the water but are present on the surfaces over which the water is flowing towards the sampling site. To get IRB to release and enter the flowing waters, it is necessary to cause a shift in the local environment that will make the conditions more hostile to the IRB. This is easily done by changing the pumping conditions (e.g., turn the pump off for a day if it is an active well) or applying a mild chemical shock using something like a low-dosage hypochlorite.

IRB infestations usually occur in the presence of oxygen and so may be more readily seen as slimes, clogs or encrustations. Over the century, these growths have had two common features: the presence of high concentration of ferric ($Fe^{+++}$) and of high populations of IRB (either as stalked *Gallionella*, the sheathed IRB or the heterotrophic IRB). The seriousness of these growths in engineered structures has led to the use of the term "Iron Bacteria". Recent research has shown that these bacteria are able to shunt the iron through oxidative and reductive states through ferric ($Fe^{+++}$) and ferrous ($Fe^{++}$) forms respectively. The BART™ biodetector is designed for the detection of these bacteria and is able to perform both the oxidative and reductive based reactions involving iron. This comprehensive group is known as the "Iron Related Bacteria" (IRB).

The medium selected for the culture of the IRB is based on an original formulation developed by Sergei Winogradsky in which the major form of iron is presented as Ferric Ammonium Citrate. The IRB-BART™ thus provides the major carbon (citrate), nitrogen (ammonium) and iron (ferric) from the same complex chemical form. When the crystallized pellet in the base of the test vial begins to dissolve after the sample has been added, a complex series of reactions occur. These reactions are influenced by both the chemical and biological composition of the sample and the redox and nutrient gradients created in the BART™ test. Under sterile conditions, a sample may be expected to cause a gradual dissolving of the nutrients from the pellet with the formation of a colored transparent diffusion front which gradually ascends through the fluid column until all of the liquid medium has a similar color. Where there has not been any major chemical reaction and the sample contains some oxygen (oxidative), the resultant color can generate yellow. If the sample is reductive (devoid of oxygen) and contains a relatively high calcium-magnesium concentration, the diffusion front may become a transparent green color.

RPS (reaction pattern signatures) revolve around a complex pattern of signals which are generated when the IRB in the water sample begin to utilize the nutrients and manipulate the ferric form of iron present in the base of the inner BART™ test vial. Common events range from:

- gas formation (common where anaerobic conditions exist),
- clouding (commonly at the REDOX (reduction-oxidation) front),
- slime formations (commonly starting at the base or around the FID ball in the test vial),
- color changes (which can pass through various shades of yellow, red, brown, to black, or through shades of green).

Careful QC is employed during manufacturing to ensure that the ferric ammonium citrate yields a consistent reproducible response to the various test cultures.

Iron related bacteria (IRB) are difficult to enumerate since they are subdivided into a number of groupings (e.g., iron oxidizing and iron reducing bacteria). These bacteria function under different REDOX conditions and utilize a variety of substrates for growth. By the routine (e.g., monthly) testing of water or wastewater using this technique, the levels of aggressivity, possible population and community structure (RPS) can all be determined. The status of an iron related bacterial population within a given sample can be determined and related to any biofouling in the surrounding environment.

To conduct the test, it is necessary to add 15mL of the sample to the biological activity reaction test biodetector. The ball floats up and restricts the entry of oxygen into the liquid medium. At the same time, components in the modified Winogradsky selective culture medium for IRB begin to diffuse upwards into the sample from a dried medium pellet in the base of the biodetector. Two gradients form within the fluid column: nutrients diffusing upwards, and oxygen diffusing downwards. These gradients form a variety of different habitats in which IRB can flourish. The color displayed by microbial activity may be a result of the form into which the ferric iron becomes modified in the medium.

It should be noted that, in a biologically active BART™ tester, the ferric form of the iron added with the selective Winogradsky medium will revert to the ferrous form along the reductive (lower) part of the redox gradient. Commonly, where there is a radical reduction of the ferric form to the ferrous during the early phase of an IRB-BART™ test, the color of the diffusing medium in the bottom of the BART™ tester may shift from a yellow to a green. This should be considered negative unless this “greening” at the base of the inner test vial is accompanied with clouding.

## 2.1 Reaction Patterns, Iron Related Bacteria

There is a range of reactions that can occur in the IRB-BART™, all of which can be observed. It is recommended that the BART™ tester be held up to a diffuse light to confirm some of these reactions which may be difficult to see against a dark background.

**BC** -Brown Cloudy
**BG** -Brown Gel
**BL** -Blackened Liquid
**BR** -Brown Ring
**CL** -Cloudy Growth
**FO** -Foam
**GC** -Green Cloudy
**RC** -Red Cloudy

Each of the reactions has been produced in a unique manner by the various species and consortia of bacteria becoming active in the test. There is therefore no specific form of any reaction pattern because these are controlled by the form of bacterial growths. Below is listed the descriptions for each of the IRB-BART™ test reactions.

**CL – Clouded Growth**
When there are populations of aerobic bacteria, the initial growth may be at the REDOX front that commonly forms above the medium diffusion front. This growth usually takes the form of lateral or "puffy" clouding which is most often grey in color. It should be noted that if the observer tips the BART™ slightly, the clouds will move to maintain position within the tube. Commonly, the medium will be darker beneath the zone of clouding and lighter above.

**BG – Brown Gel**
In this reaction, a basal, gel-like brown growth forms that maintains structure and position even when gently rotated or tilted. This brown gel can occupy the whole of the basal cone of the inner test vial and also extend up the sidewall of the inner test vial to a height of <15 mm. The solution above the gel is commonly clear and colorless. Over time it is often noticed that the size of the gel mass will grow and later shrink. Detachment sometimes happens so that a single brown gel-like mass can be seen floating in the test vial.

**BC – Brown Cloudy**
Unless there is a very large population of IRB in the sample, this reaction is normally a secondary reaction (often following reactions CL, FO, or RC) and may be recognized as a dirty brown solution that may have a brown ring around the ball.

**FO – Foam**
This is a very easy reaction to recognize since gas bubbles around the ball form a foam ring or sometimes the bubbles collect over greater than 50% of the underside of the ball. On some occasions, bubbles will collect on the walls of the inner test vial but is not significant until the bubbles collect around the ball. The solution usually remains clear but commonly has a yellow or greenish-yellow color. The bubbles can sometimes be seen in the foam to be individually coated with slime that may give the bubbles a color ranging from brown through to orange, yellow or grey. Sometimes when integrated together into a foam, this foam is tough enough to either "lift" the FID out of the liquid solution or submerge the FID below the surface of the liquid solution.

Do not confuse this reaction with the generation of bubbles (usually randomly) when oxygen supersaturates as the sample temperature comes up from a lower temperature (of the sample's source). These bubbles are recognized as being reflective and not bound in any slime and dispersed within the inner test vial under the ball and on the walls. They usually disappear within two days.

This FO reaction is most commonly related to a sample in which many microbes are functioning anaerobically. It can often be "harmonized" with the presence of SRB (reactions BB, BT or BA). In other words, the occurrence of a FO in the IRB-BART™ can often be followed by a positive detection of SRB in the SRB-BART™ if that test has been performed on the same sample.

**RC – Red, Slightly Clouded**
The liquid medium remains a clear to a dark reddish solution. The solution will cloud fairly quickly and shift to a BC reaction generally after a BR has formed around the ball.

**BR – Brown Ring**
A reddish- brown to dark brown slime ring forms around the ball. This ring is entire and tight and usually <3 mm in width. Generally, the brown slime ring will sit between the liquid surface and the equator of the ball and commonly intensifies over time. On some occasions this reaction possesses unusual feature in that the slime ring can "bio-lock" the ball to the walls of the test vial. In these cases, when the test vial is turned upside down, the ball remains (glued) in-place and the liquid remains above the ball. What has happened is that the ring has become formed biologically into a hydraulic barrier.

**GC – Green Clouded**
Solution goes to a shade of green and becomes cloudy without, necessarily, the formation of defined clouds or gel-like forms. No slime ring is formed around the FID. This cloudiness will gradually increase and often this reaction will shift to a dark green very cloudy solution. As the solution becomes a darker green and cloudier, a BR reaction may form but this is usually fairly thin.

**BL – Blackened Liquid**
This is commonly a secondary or tertiary reaction rather than an initial reaction. It is recognized as a clear, often colorless, solution surrounded by large blackened zones in the basal cone and up the walls of the inner test vial.

Other reactions not coded are described below. These reactions occur less than 1% of the time in water testing using the IRB-BART™:

"Fuzzy" growths around the ball, IRB-BART™, occasionally where a water sample has traveled through a semi-saturated zone, there are fungal spores present. These create reaction thirteen in which a white, grey or speckled "fuzzy" mat forms around and even over the ball. The upper surface of the mat often forms into a tight mass with an irregular surface. The lower surface of the mat can often be seen to be extending into the liquid medium by thread-like processes 2 to 5 mm in length. These growths may bio-lock the ball to the wall of the inner test vial for a period of time. Solution usually remains fairly clear but globular-like deposits may be present. Solution may cloud over time. This reaction is caused by the presence of large populations of fungal spores in the water.

## 2.2 RPS (Reaction Pattern Signatures) for the IRB-BART™

Because of the complex communities that form the iron bacteria, the reaction patterns can develop some very distinctive sequences. In the last ten years, the meaning of the sequences (RPS) has been determined. The common characterizations are listed below:

- **BC – WB – BR** IRB with carbonate deposition and some slime formers present
- **CL – GC** Mixed heterotrophic IRB dominated by Pseudomonads
- **CL – BG** Mixed heterotrophic IRB with some Enteric bacteria (possibly *Enterobacter*)
- **CL – BC** Mixed heterotrophic IRB
- **CL – BC – BR** Mixed heterotrophic IRB with some slime formers
- **CL – FO** IRB with mixed aerobes and some anaerobic activity
- **CL – BC** A white deposit forms in the vial. Aerobic IRB with carbonate deposition
- **FO – CL** Anaerobic bacteria with some aerobic heterotrophic IRB
- **FO – CL – RC** Anaerobic bacteria with some aerobic heterotrophic IRB and Enteric bacteria (possibly *Enterobacter, Citrobacter* or *Serratia*)
- **FO – CL – BC – BR** Mixed anaerobic and Enteric bacteria with some slime forming IRB
- **FO – BR – BC** Mixed anaerobic and IRB with some aerobic slime forming bacteria
- **FO – GC** Mixed anaerobic and aerobic bacteria dominated by Pseudomonads
- **FO – GC – BL** Mixed anaerobes, Pseudomonads and Enteric bacteria
- **GC** Most of the bacteria present are Pseudomonads
- **GC – BL** Pseudomonads dominate with some IRB and Enteric bacteria present
- **RC – CL – BR** Enteric bacteria dominate

The IRB are generally slow growing and often will display the first reaction as either a foam (FO) or a cloudy plate (CP). The consortium is complex and involves a mixture of stalked and sheathed bacteria along with heterotrophic and slime forming bacteria. Because of the complex nature of this consortium, it takes longer to become established and is more likely to show a succession of secondary reactions as the consortium stabilizes.

## 2.3 Time Lag (days of delay) to IRB-BART™ Populations

The populations of IRB can be determined using the time lag to the observation of the first reaction. This relationship is shown in Table Four.

**Table Two**

**The Relationship between Time Lag and the Population For Iron Related Bacteria**

| Time Lag (days) | Population cfu/ml |
|---|---|
| 1 | 540,000 |
| 2 | 140,000 |
| 3 | 35,000 |
| 4 | 9000 |
| 5 | 2300 |
| 6 | 500 |
| 7 | 150 |
| 8 | 25 |

## 2.4 Risk Potential Assessment – IRB-BART™

The IRB are a complex of many bacteria that possess a common ability to utilize iron. As a result this test has a complex set of reactions which can be displayed. The shorter the time lag to the IRB displaying a reaction, the greater the aggressivity and the need to treat. Not all reactions are equally important in determining the aggressivity of the IRB (and therefore the need to treat). Below is a list of the reactions described previously and the relative importance in relation to the need to treat. Concern can be expressed through the shortness of the time lag (in days) as:

1-2. Very aggressive (treatment should be started as early as convenient)
2-4. Aggressive (treatment should be considered in the near future before the condition degenerates further)
5-8. Moderately Aggressive (treatment may not be required but vigilance through ongoing testing should be practiced)

5-9. >8. Normal Background Levels (routine testing is recommended)

**Table Three**

**Relationship between the Time Lag to the First reaction in an IRB-BART™ and the Aggressivity of the Iron Related Bacteria**

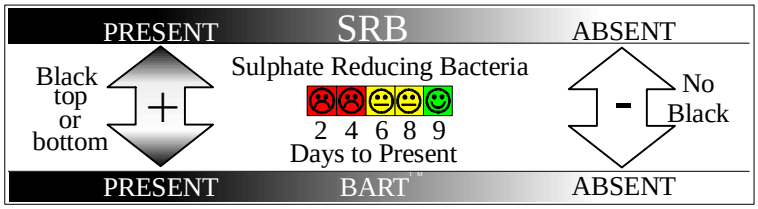
| | | Aggressivity | | | |
|---|---|---|---|---|---|
| | | Very | Sign. | Moderate | Not |
| **BC** | -Brown Cloudy | <2 | 3 | 4-8 | >8 |
| **BG** | -Brown Gel | <1 | 2-6 | 7-8 | >8 |
| **BL** | -Blackened Liquid | <2 | 3-6 | 7-8 | >8 |
| **BR** | -Brown Ring | <1 | 2 | 3-6 | >6 |
| **CL** | -Cloudy Growth | <0.5 | 0.5-2 | 3-4 | >4 |
| **FO** | -Foam | <0.5 | 0.5-1 | 2-4 | >4 |
| **GC** | -Green Cloudy | <1 | 2-4 | 5-8 | >8 |
| **RC** | -Red Cloudy | <1 | 2-3 | 4-8 | >8 |

Some remedial treatments should be considered urgently where the time lag (in days) shows aggressivity to be at the 1 or 2 level. Where there has been a RPS (sequence of reactions to form a signature), then the aggressivity should be considered to be equivalent to the most aggressive of the reactions using the above table.

## 2.5 Hygiene Risk Considerations

Four of the possible reactions can indicate a potential hygiene risk. These include: BG, BL, GC. and RC. Where these are found to have a time lag that would project an aggressivity of 1 (very aggressive) or 2 (aggressive), then a fecal coliform test should be performed to ensure that there were no fecal coliform bacteria present. Note that the use of the total coliform test could yield a positive since some of the bacteria causing these reactions could be environmental enterics. If the RPS includes GC, a test for the presence of fluorescing Pseudomonads should also be performed.

## 3.0 SULFATE REDUCING BACTERIA, SRB-BART™




Sulfate reducing bacteria (SRB) are a group of anaerobic bacteria that, as a part of their normal activities, generate hydrogen sulfide ($H_2S$). This product can cause a number of significant problems. These range from "rotten egg" odors, through to the blackening of equipment, waters and slime formations, and the initiation of corrosive processes.

Detection of these microorganisms is made more challenging because they are anaerobic and tend to grow deep within biofilms (slimes) as a part of a microbial community (consortium). Detection of the SRB is therefore made difficult because SRB may not be present in the free-flowing liquid over the site of the fouling but are growing deeper down in the biofilms. Because of this, the symptoms of SRB fouling may precede their detection using the SRB-BART™ unless a successful attempt is made to disrupt these biofilms and cause the SRB to come up into the liquid.

The sulfate reducing bacteria are an unusual group in that they utilize hydrogen rather than oxygen as the basic driver for many of the metabolic activities. As a result of this, the SRB are anaerobic and are inhibited by the presence of oxygen. Sulfate reduction appears to be coupled to the formation of ATP (a major energy driver in metabolism) by a proton motive force (PMF) derived from electron transport. The bottom line is that the sulfate is reduced in a step-wise fashion to $H_2S$ while releasing energy for growth. It is the $H_2S$ which creates the problems through electrolytic corrosion, "rotten" egg smells, bad taste problems and the formation of black slimes.

There is another group of SRB which cause the reduction of sulfur to $H_2S$ but these are not detected using the SRB-BART™. Usually, these sulfur-reducing bacteria are less common and, hence, have been discounted in the SRB-BART™ tester. Upon special request, there is a tester for the sulfur reducing bacteria (SRB-BART™) which can be made to special order.

SRB activity in the BART™ tester is easily recognized since the sulfate becomes reduced to hydrogen sulfide. This product now reacts with the diffusing ferrous iron to form black iron sulfides. This sulfide commonly forms either in the base (as black precipitates) and/or around the ball (as an irregular black ring). In the latter event, the SRB may form a part of an aerobic consortium forming around and on the FID ball. Generally, where this happens, the blackening may be seen as granular structures held within the slime ring that is commonly not totally black.

The SRB-BART™ uses the short chain fatty acids to provide the substrates for the growth of the SRB. On some occasions, heterotrophic anaerobic bacteria can also become very active in the BART™ test and often grow faster than the SRB. When this happens, the liquid will tend to go cloudy. Usually, this is seen as a gel-like clouding most commonly in the bottom third of the BART™ inner test vial and shows that anaerobic heterotrophs are present and active. It should be remembered that these bacteria might not necessarily grow in the SLYM-BART™ since the major organic carbon nutrients are not short chain fatty acids.

Under exceptional circumstances, an SRB-BART™ may display a blackening very quickly (e.g., less than half an hour). In this case it is likely that the sample being tested contains some residual hydrogen sulfide which has rapidly reacted with the iron in the test vial. Where this happens, it is recommended that the water sample be aseptically aerated to drive off the gaseous hydrogen sulfide

from the sample before conducting the SRB-BART™ test. While the aeration would admit oxygen to the sample, the SRB should survive through being protected by the other bacteria within the slime formations.

## 3.1. Reaction Patterns, Sulfate Reducing Bacteria

**BB** - Blackened Base
**BT** - Blackening around Ball

There are three reaction patterns that are positive for the SRB. Detailed descriptions of these is given below:

**BB – Blackened Base**
The reaction is recognizable by the formation of a blackened deposit in the basal cone of the test vial. It may be first observed by looking up into the underside of the cone of the inner test vial. Blackening frequently starts as a 2 to 3-mm wide ring around the central peg and gradually spreads outwards. Black specking may also occur on the bottom 15 mm of the walls of the test vial immediately above the cone. The liquid medium should be clear (see reaction CG below) and there should be no slime ring around the ball.

**BT – Blackening around the Ball**
A slime ring may be viewed around the ball with patches of black specking or zones intertwined in the slime growths. The slime itself is not a characteristic of this reaction but the blackening is. The slime usually is either a white, grey, beige, or yellow color and tends to form on the upper side of the ball. The blackening often begins as a specking which gradually expands to patches within the slime.

**Combination of BB and BT**
A combination of reactions BB and BT constitute a reaction BA. Blackening occurs both in the base and around the ball although the length of the inner test vial may not be blackened.

The other recognized reaction is a negative for SRB but commonly occurs where there are aggressive anaerobic bacteria present. Often this reaction will precede a positive reaction for SRB (i.e., BB and BT). This negative SRB reaction is:

**CG – Cloudy Gel-Like**
While not a positive indication for the presence of SRB, this reaction is recognized since it does indicate the presence of anaerobic bacteria and often precedes the generation reactions BB, BT or BA. It is recognized by the appearance of cloud-like structures in the colorless liquid medium. Usually these form from the bottom up and initially at a height of 20 to 25 mm up the sidewall of the inner test vial. This clouded zone may expand to render the liquid medium turbid. These clouds are relatively stable structures and have defined edges.

## 3.2.RPS (Reaction Pattern Signatures) for the SRB-BART™

- **BB** Deep-seated anaerobic bacteria dominated by *Desulfovibrio*
- **BT** Dominant aerobic slime forming heterotrophs include SRB in the consortium
- **BB – BA** Dominant anaerobic consortium including SRB with a fraction able to function aerobically as slime formers incorporating the SRB
- **BT – BA** Aerobic slime formers incorporate SRB and are also able to colonize anaerobic

Conditions

Note that the SRB-BART™ includes another common test reaction, which does not relate to the presence or absence of the SRB in the sample under test. This test reaction is recognized by the development of a cloudy growth that often begins close to the base and gradually fills at least 20% of the liquid volume. Often this growth reaction appears almost gel-like and has a fuzzy but distinct edge. This may be admitted as a reaction:

**CG** - Cloudy Gel-like

This reaction does not mean that SRB are present but that anaerobic bacteria are. Commonly, the **CG** reaction precedes the blackening or occurs shortly after the commencement of the blackening.

## 3.3. Time Lag (days of delay) to SRB-BART™ Populations

The common relationship between the time lag measured in days of delay and the population of SRB is given in Table Six. Because the SRB commonly are aggressive as a part of a consortium of different species of bacteria, their numbers may be difficult to determine using some of the standard procedures for SRB. This methodology allows the growth of the consortium in the SRB-BART™ that, consequently, initiates greater levels of aggressivity. The populations given in Table Six reflect the higher recovery rates and comparisons with other tests may show the SRB-BART™ to be the more sensitive.

**Table Four**

**The Relationship between Time Lag and the Population For Sulfate Reducing Bacteria**

| Time Lag (days) | Population cfu/ml |
|---|---|
| 1 | 6,800,000 |
| 2 | 700,000 |
| 3 | 100,000 |
| 4 | 18,000 |
| 5 | 5000 |
| 6 | 1200 |
| 7 | 500 |
| 8 | 200 |

Sulfate reducing bacteria (SRB) are a narrow group of bacteria that have the common facility to reduce sulfates to hydrogen sulfide. It is this sulfide which reacts with metals (commonly iron) to form the black sulfides. It is these black deposits that cause an identifiable reaction in the base of the tube (BB) or around the ball (BT). In both cases, the SRB do function as part of a consortium that is either anaerobic (BB) or aerobic (BT).

### 3.4. Risk Potential Assessment-SRB-BART™

The SRB are a relatively simple consortium in which the SRB tend to either dominate over a facultative/strict anaerobic heterotrophic bacterial flora (BB), or become integrated into an aerobic slime forming heterotrophic bacterial community growing around the ball (BT). Where a more complex and aggressive form of SRB are present (involving both forms of consortial activity BA), then the SRB are usually very aggressive and the BA reaction occurs without being preceded by either of the other two reactions. The risk potential for the severity of a detected SRB event can be expressed through the shortness of the time lag (in days) as follows:

1. Very aggressive (treatment should be started as early as convenient)
2. Aggressive (treatment should be considered in the near future before the condition degenerates further)
3. Moderately Aggressive (treatment may not be required but vigilance through ongoing testing should be practiced)
4. Normal Background Levels (routine testing is recommended)

**Table Five**

**Relationship between the Time Lag to the First Reaction in an SRB-BART™ and the Aggressivity of the Sulfate Reducing Bacteria**

| | | | Aggressivity | | | |
|---|---|---|---|---|---|---|
| | | | Very | Sign. | Moderate | Not |
| BB | - | Black Base | <1 | 2-3 | 4-8 | >8 |
| BT | - | Black Ball | <1 | 2-4 | 5-8 | >8 |
| BA* | - | Black All | <2 | 2-5 | 6-8 | >8 |

Note: The BA reaction (*) listed above must have occurred without either of the other reactions occurring first. If either the BB or the BT reaction did occur first, then the aggressivity should be based on the first of the reactions that did occur. Some remedial treatments should be considered urgently where the time lag (in days) shows aggressivity to be at the 1 or 2 level.

A non-SRB reaction can also commonly occur in this test when a cloudy gel (CG) forms. This is indicative of the presence of anaerobic bacteria (not SRB). However, on some occasions, these anaerobic bacteria can also become very aggressive and can cause deep-seated plugging. The aggressivity for these bacteria can be judged using the table below:

**Table Six**

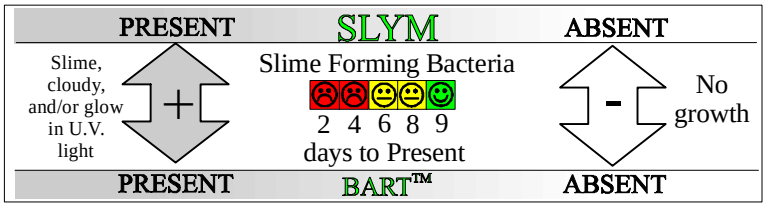
**Relationship between the Time Lag to the CG Reaction in an SRB-BART™ and the Aggressivity of the Anaerobic Bacteria**

| | | | Aggressivity | | | |
|---|---|---|---|---|---|---|
| | | | Very | Sign. | Moderate | Not |
| CG | - | Cloudy Growth | <0.5 | 0.5-2 | 3-4 | >4 |

### 3.5. Technical Advisory

The advisory notifies users of the SRB-BART system for the detection of sulfate reducing bacteria that the standard maximum length for the monitoring of the reaction patterns is commonly ten (10) days. Operators using the SRB-BART tester for the detection of deep-seated SRB infestations in water systems associated with wells and distribution systems may find it advantageous to continue observations until the fifteenth ($15^{th}$) day. This is because some SRB do not exhibit reaction patterns (i.e. BT or BB) until after other bacterial consortia have already grown within the tester (e.g. anaerobic bacteria). This delays the observation of a positive detection for the SRB. In water pipelines and biofouling water wells the time lags can be delayed until days 11 to 15. It is not possible to project the size of the SRB populations but this extension of the testing period can be used to determine the presence/absence of the SRB when they are present in the environments either in very low numbers or in a consortial association with other microbial species. It can be expected that where routine monitoring is being undertaken, sudden decreases in the time lags to 10 days or less can be taken to indicate that the SRB are becoming significantly more aggressive and may require corrective action (e.g. disinfection, pigging the lines, etc.).

## 4.0 SLIME FORMING BACTERIA, SLYM-BART™




Slime-Forming Bacteria (SLYM) is the name given to bacteria that are able to produce copious amounts of slime without necessarily having to accumulate any iron. These slime-like growths are therefore often not dominated by the yellows, reds and browns commonly seen where IRB are present. Some of the IRB also produce slime but it is sometimes denser and has more texture due to the accumulation of various forms of insoluble iron. SLYM bacteria can also function under different reduction-oxidation (REDOX) conditions but generally produce the thickest slime formations under aerobic (oxidative) conditions. These can develop in the SLYM-BART™ as slime rings growing around the floating ball. Slime growth can also be seen as a cloudy (fluffy or tight plate-like structures) or as gel-like growths which may be localized or occur generally through the body of water medium. Very commonly the gel-like slime growths form from the bottom up in the test vials. One common check for these types of growth is to tilt the BART™ gently and see that the cloud- or gel- like growths retain their structure and tilt with the tube.

A vast majority of bacteria can produce slime-like growths. The slime is actually formed by a variety of exopolysaccharide polymers that are long thread like molecules. These extracellular polymeric substances (EPS) literally coat the cells into a common slime-mass within which large volumes of water become clustered and bound. Often 95 to 99% of the volume of slime are actually water. Some bacteria produce an EPS that remains tightly bound to the individual cell. These are called capsules. Other bacteria generate such a copious amount of EPS that it envelops whole masses of cells within a common slime.

The role of the slime appears to be protective. If environmental conditions are harsh (e.g., due to shortage of nutrients), the slime layers tend to get thicker. Not only does the slime act as a protectant to the resident bacteria but it also acts as a bio-sponge by accumulating many chemicals that could form either a nutrient base, or be toxic to the cells. EPS may be produced by enzymatic activity (e.g., dextran sucrase or levan sucrase) on carbohydrates. In addition, EPS may be synthesized within the bacterial cells and released to form an enveloping slime.

Slime forming bacteria tend to be aerobic and form slimes at REDOX fronts. In the BART™ tester, this front may form around the ball causing a slime ring, or deeper down in the liquid medium column to form an observable growth. This growth may be plate-like and appear to float at a specific depth, cloud-like with indefinite edges, form as basal dense slimes in the conical base of the test vial, or be gel-like and maintain its shape even when the vial is tilted. Since slime tends to be formed by bacteria under stress, it is common for the slimes to form after there has been an initial growth that may take the form of a localized or general cloudiness.

Many slime bacteria can produce various pigments that will color the slime. Such growths are usually white, grey, yellow or beige in color. These often darken over time particularly in the presence of daylight. Distinctive colored slimes include red (commonly associated with *Serratia marcescens*) and violet (associated with either *Chromobacterium* or *Janthinobacterium* species). Blackening may also occur particularly after growth. This may be a result of the production of either

iron sulfides or carbonates which is commonly associated with the presence of mixed cultures including enteric bacteria in the SLYM-BART™.

SLYM-BART™ can be used as a simple presence/absence (P/A) test capable of indicating to some extent the population size and the types of SLYM organisms present in the sample. Different microorganisms utilize various sites along the REDOX gradient under the ball to grow and regular careful observations are needed to catch the start of growth so that the time lag can be determined.

Slime forming bacteria cause very serious engineering problems since the slime formation can compromise the engineered specifications into many systems. Primarily, the effects of the slime growths are to reduce hydraulic or thermal conductivity and reduce water quality (generally, the first symptom is increased turbidity followed by taste, odor or color problems. As the slimes slough into the fluid later during the infestation, it can be expected to see sudden rises in the total organic carbon, increases in aggressivity and reductions in water quality.

## 4.1 Reaction Patterns, Slime Forming Bacteria

**DS** -Dense Slime (Gel-Like)
**SR** -Slime Ring around the Ball
**CP** -Cloudy Plates layering
**CL** -Cloudy Growth
**BL** -Blackened Liquid
**TH** -Thread-Like Strands
**PB** -Pale Blue Glow in U.V. Light
**GY** -Greenish-Yellow Glow in U.V.

Of the above reactions, it is the CL (cloudy) reaction that is by far the most common. Often the CL will be preceded by a CP which will be transient (lasting commonly less than 24 hours). Descriptions of the various reaction is given below:

**DS – Dense Slime**
This reaction may not be obvious and require the observer to gently rotate the BART™ test at which time slimy deposits swirl up. These deposits may swirl in the form of a twisting slime when the tube is gently rotated. This swirl can reach 40 mm up into the liquid column, or it may rise up as globular gel-like masses that settle fairly quickly. Once the swirl has settled down, the liquid may become clear again. In the latter case, care should be taken to confirm that the artifact is biological (ill-defined edge, mucoid, globular) rather than chemical (defined edge, crystalline, often white or translucent). Generally, these dense slime growths are beige, white or yellowish-orange in color.

**CP – Cloudy Plates Layering**
When there are populations of aerobic bacteria, the initial growth may be at the REDOX front that commonly forms above the yellowish-brown diffusion front. This growth usually takes the form of lateral or "puffy" clouding which is most commonly grey in color. Often the lateral clouds may be disc-like in shape (plates) and relatively thin (1 to 2 mm). It should be noted that if the observer tips the BART™ slightly, the clouds or plates often move to maintain position within the tube. The edges of the plates are distinct while the edges of the “puffy” forms of layering are indistinct. These formations are most commonly observed 15 to 30 mm beneath the fill line. While cloud formations will tend to extend to cause an overall cloudiness of the liquid medium (CL). These plates sometimes appear to divide (multiple plating) before coalescing into a cloudy liquid medium.

**SR - Slime Ring**
A slime ring, usually 2 to 5 mm in width forms on the upper side of the ball. The appearance is commonly mucoid and may be a white, beige, yellow, orange or violet color that commonly becomes more intense over time on the upper edge.

**CL – Cloudy Growth**
Solution is very cloudy and there may sometimes be a poorly defined slime growth around the ball. Sometimes a glowing may be noticed in at least a part of the top 18mm of the liquid medium. This glowing is due to the generation of U.V. fluorescent pigments by some species of *Pseudomonas*. The common pigments doing this are a pale blue (PB) or a yellowish green (YG) color. Note that this glowing may not be readily observable unless a U.V. light is used. The occurrence of the glowing in a U.V. light means that there is a probability of potentially pathogenic species of *Pseudomonas* and confirmatory testing is recommended.

**BL – Blackened Liquid**
This is commonly a secondary or tertiary reaction rather than an initial reaction. It is recognized as a clear, often colorless, solution that is surrounded by large blackened zones in the basal cone and up the walls of the test vial. The BL often parallels the BL reaction in the IRB when the two BARTs™ are used together to test the same sample.

**TH – Thread-Like Strands**
On some occasions, the slime forms into threads that form web-like patterns in the liquid medium. Sometimes these threads which interconnect from the ball to the floor of the inner test vial.

## 4.2 RPS (Reaction Pattern Signatures) for the SLYM-BART™

- **DS - CL** Dense slime forming bacteria producing copious EPS, facultative anaerobes dominate
- **SR - CL** Aerobic slime forming bacteria (such as M*icrococcus*) dominating with some facultative anaerobes
- **CP - CL** Motile facultatively anaerobic bacteria dominate (e.g., *Proteus*)
- **CL - SR** Mixed bacterial flora including some aerobic slime-formers
- **CL - BL** Slime formers dominated by Pseudomonads and Enteric bacteria
- **CL - PB** *Pseudomonas aeruginosa* dominant member of the bacterial flora
- **CL - GY** *Pseudomonas fluorescens* species group present in the flora
- **TH - CL** Aerobic bacteria dominant which are able to generate slime threads (e.g., *Zoogloea*)

## 4.3 Time Lag (days of delay) to SLYM-BART™ Populations

The slime forming bacteria are among the fastest growing aggressive consortia and the medium used in this BART™ is very enriching and causes a wide variety of bacteria to grow rapidly. However, when the bacteria do not grow quickly this indicates a very low population of aggressive bacteria. As a result of this, the time lags of between 3 and 6 days show a rapid decline in populations when compared to the IRB or SRB BART™ tests.

**Table Seven**

**The Relationship between Time Lag and the Population For Slime Forming Bacteria**

| Time Lag (days) | Population cfu/ml |
|---|---|
| 1 | 1,800,000 |
| 2 | 350,000 |
| 3 | 66,500 |
| 4 | 12,500 |
| 5 | 2500 |
| 6 | 500 |
| 7 | 100 |
| 8 | 10 |

## 4.4 Risk Potential Assessment – SLYM-BART™

The slime forming bacteria are complex consortia involving many bacteria. These consortia inhabit a common "growth" of slime that acts as a communal chamber. Within these slimes, the bacterial cells are commonly dispersed and occupy only a small part of the total volume (<0.1%). Most of the slime is water bound to the organic polymers that bind the slime together. The SLYM-BART™ reflects the activities of bacteria that are present in the water as a result of the sloughing from the slime. As a result of this, the test may exhibit a complex set of reactions depending upon precisely which bacterial species are present in the sample. Like the other BART™ tests, the shorter the time lag to the SLYM-BART™ displaying a reaction then the greater becomes the aggressivity and the more urgent the need to treat. Not all reactions are equally important in determining the aggressivity of the slime forming bacteria (and therefore the need to treat). Below are a list of the reactions described above and their relative importance in relation to the need to treat. Concern can be expressed through the shortness of the time lag (in days) as:

1. Very aggressive (treatment should be started as early as convenient)
2. Aggressive (treatment should be considered in the near future before the condition degenerates further)
3. Moderately Aggressive (treatment may not be required but vigilance through ongoing testing should be practiced)
4. Normal Background Levels (routine testing is recommended)

**Table Eight**

**Relationship between the Time Lag to the Reactions in a SLYM-BART™ and the Aggressivity of the Slime Forming Bacteria**

| | | Aggressivity | | | |
|---|---|---|---|---|---|
| | | Very | Sign. | Moderate | Not |
| **DS** | -Dense Gel Slime | <1 | 2 | 3-7 | >7 |
| **SR** | -Slime Ring | <1 | 2-3 | 4-6 | >6 |
| **CP** | -Cloudy Plates | <0.5 | 1-2 | 3-6 | >6 |
| **CL** | -Cloudy Growth | <1 | 2 | 3-6 | >6 |
| **BL** | -Blackened Liquid | <1 | 2-4 | 5-8 | >8 |
| **TH** | -Threads | <2 | 3-4 | 5 | >6 |
| **PB** | -Pale Blue Glow | <1 | 2-4 | 5-8 | >8 |
| **GY** | -Green-Yellow Glow | <1 | 2-3 | 4-8 | >8 |

Some remedial treatments should be considered urgently where the time lag (in days) shows aggressivity to be at the very aggressive or aggressive (1 or 2) levels. Where there has been a RPS (sequence of reactions to form a signature), then the aggressivity should be considered to be equivalent to the most aggressive of the reactions using the above table.

## 4.5. Hygiene Risk Considerations

The most significant hygiene risk generated by this test is the BL reaction that indicates that Pseudomonads and enteric bacteria are present. If this reaction occurs within eight days then a fecal coliform test should be performed on that water to determine the hygiene risk directly. Where PB or GY reactions are observed, this should be confirmed using the FLOR-BART™.

## 5.0 HETEROTROPHIC AEROBIC BACTERIA, HAB-BART™


Some bacteria are able to degrade organics as their source of energy and carbon. These are known as heterotrophic bacteria. By far, the majority of these heterotrophs function most efficiently under aerobic conditions. Much of the biodegradation that occurs under aerobic conditions is due to the activities of these heterotrophic aerobic bacteria (HAB, formerly the total aerobic bacteria or TAB). Since these bacteria play a major role of biodegradation and their presence in oxygen-rich environments can be critical to the efficiency of the engineered operation, the HAB-BART™ was developed to detect these bacteria.

The unique feature of this test is the addition of methylene blue that acts as an indicator of respiratory activity. While there remains free oxygen in the sample, the methylene blue dye in the liquid medium remains blue. As soon as all of the oxygen has been consumed by bacterial (respiratory) activity, the methylene blue shifts from its observable form to a colorless form. In other words, in the HAB-BART™ tests, when the liquid medium turns from blue to a colorless (non-blue) form, then the heterotrophic aerobic bacteria have been sufficiently aggressive to have “respired off” the oxygen. At this time a methylene blue reductase enzyme becomes activated and this reduces the methylene blue to its colorless form.

Microorganisms present at depths in this test are short of oxygen and "look" for alternatives. The blue dye (methylene blue) in this test forms such an alternate substrate. When the aerobic bacteria use this dye, the color is bleached out. This usually occurs from the bottom (bottom up) or the top (top down) of the tester first. This bleaching action (decolorizing the blue dye) is the indicator of a positive reaction. Note that the dye is added to the test by inverting the charged HAB-BART™ for 30 seconds to allow the methylene blue chemical dried in the cap time to dissolve into the sample. When the HAB-BART™ is returned to its normal state (cap side up), the ball rolls up through the liquid medium causing the methylene blue to become mixed into the sample to form an even blue solution.

Methylene blue is a basic dye that can bind readily to the negatively charged microbial cells. Traditionally, therefore, this dye has been used to stain microbial cells. A feature of methylene blue is that it changes from a blue color in the oxidized state to a clear form in the reduced state. When methylene blue is added to a medium that is actively converting energy due to microbial respiration, the electrons are transferred to the dye causing it to become reduced and the dye changes from a blue to a clear state (the color disappears). The protocol has been based on the methylene blue reductase test that has been used in the dairy industry for decades to determine the potential for bacterial spoilage of milk. In the HAB-BART™ the objective is for the user to be able to determine the aerobic bacterial population which may be related to various forms of biofouling and bioremediation. Essentially, the methylene acts as an oxygen substitute and its reduction (bleaching) from the blue to the colorless form can be used an indication of the amount of respiratory function of the bacteria in the sample.

This test is therefore an answer to the need to test water and wastewater for the presence of heterotrophic aerobic bacteria as such without trying to determine the particular groups of bacteria that may be present.

The HAB-BART™ determines the activity of the Heterotrophic Aerobic Bacteria. When these bacteria are present and active, the blue dye in the biodetector becomes bleached (colorless) either

from the bottom up or the top down. The faster this happens, the more aggressive are the heterotrophic aerobic bacteria.

## 5.1 Reaction Patterns, Heterotrophic Aerobic Bacteria

**UP** -Bleaching moves upward from base
**DO** -Bleaching moves downward from ball

There are only two recognized reactions (UP and DO) and both of these relate to the form with which the bleaching occurs. There are different forms of clouding which follow the bleaching of the methylene blue and these are recognized using the BARTSCAN™ system.

**UP – Bleaching moves upwards**
Blue solution bleaches from the bottom up. The bleached zone may be clear or clouded. In the latter case, the medium tends to have a light to medium yellow color. Rarely does the bleaching extend beyond the equator of the ball so that a blue ring will remain around the ball with a width of 1 to 5 mm.

**DO – Bleaching moves downwards**
Blue solution bleaches from the top down. The bleached zone is more commonly cloudy. The bleached liquid medium tends to have a light to medium yellow color. Commonly the bleaching does extend up beyond the equator of the ball and any blue ring remaining around the ball is relatively thin with a width of 0.5 to 2 mm.
**Note**: that there is almost always a blue ring remaining around the ball and that the DO reaction will usually leave this ring intact. Furthermore, the test reaction can only be one or the other and so interpretation is restricted to one or other of these two reactions.

## 5.2 RPS (Reaction Pattern Signatures) for the HAB-BART™

- **UP** Strictly aerobic bacteria may be dominant with some facultative anaerobes often present
- **DO** Facultatively anaerobic heterotrophs dominate along with some anaerobic bacteria

## 5.3 Time Lag (days of delay) to HAB-BART™ Populations

The relationship between the time lag (days of delay) to the bacterial population is given in Table Eleven.

**Table Nine**

**The Relationship between Time Lag and the Population For Heterotrophic Aerobic Bacteria**

| Time Lag (days) | Population cfu/ml |
|---|---|
| 1 | 7,000,000 |
| 2 | 500,000 |
| 3 | 50,000 |
| 4 | 7000 |

The heterotrophic aerobic bacteria, like the slime formers, grow very quickly and are readily detectable because of the reduction of the methylene blue from the blue (oxidative) to the

colorless (reductive) state. Essentially, the methylene blue acts as a redox indicator and rapidly shows when respiratory activity is occurring because the test liquids become reductive and the methylene blue decolorizes. This test is one of the fastest of the BART™ tests as well as being the easiest to read. It functions most effectively when the bacterial consortia in the sample are dominated by heterotrophic aerobes.

## 5.4 Risk Potential Assessment – HAB-BART™

The heterotrophic aerobic bacteria are subdivided into two major consortial groups in the HAB-BART™. These are dominated by either: the strictly aerobic (UP), or the facultatively anaerobic (DO) heterotrophic bacteria. The risk potential for the severity of a detected HAB event can be expressed through the shortness of the time lag (in days) as follows:

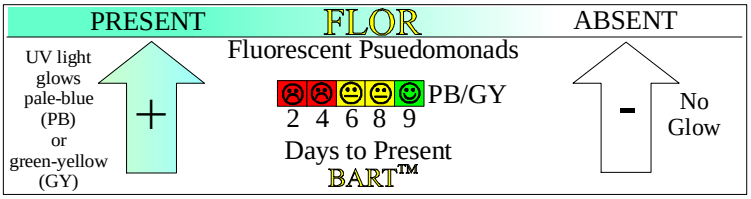
1. Very aggressive (treatment should be started as early as convenient)
2. Aggressive (treatment should be considered in the near future before the condition degenerates further)
3. Moderately Aggressive (treatment may not be required but vigilance through ongoing testing should be practiced)
4. Normal Background Levels (routine testing is recommended)

**Table Ten**

**Relationship between the Time Lag to the Reactions in an HAB-BART™ and the Aggressivity of the Heterotrophic Aerobic Bacteria**

| | | Aggressivity | | | |
|---|---|---|---|---|---|
| | | Very | Sign. | Moderate | Not |
| **UP** | -Bleach Up | <0.5 | 1-2 | 3-4 | >4 |
| **DO** | -Bleach Down | <1 | 2-3 | 4-6 | >6 |

## 6.0 FLUORESCING PSEUDOMONADS, FLOR-BART™




The Pseudomonads are a very important group of Gram negative bacteria that are found in very substantial numbers in soils, waters and many other natural materials. In associations with many plants and animals, the Pseudomonads can act as agents for disease. In aerobic bioremediation and biodegradation processes, members of the Pseudomonads often play critical roles in the biochemical breakdown of critical organic compounds. These various important aspects have led to the development of the FLOR-BART™ which generate conditions favorable to the growth of the Pseudomonads.

One critical aspect of this biodetector is the ability to generate soluble fluorescent pigments when some species of the genus *Pseudomonas* are dominant in the water. These pigments are usually produced after growth has occurred and generally can be detected most easily in the culture medium around the ball (top 20mm of the liquid column). Detection is by the use of an ultra violet (UV) lamp aimed at the top one third of the liquid column in the FLOR-BART™. Maximum excitation of these fluorescing molecules is at 400nm. There are two main pigments, pyocyanin and pyoverdins. Pyocyanin is a distinctive pigment that fluoresces with a pale blue to blue color and is most commonly associated with the species, *Pseudomonas aeruginosa*. This species is commonly associated with clinical specimens (wounds, burns, otitis, sepsis, pneumonia, urinary tract infections), a condition known as "blue pus", and is a hygiene concern in recreational waters. Pyoverdins is the name given to a group of other fluorescent pigments generated by different species of *Pseudomonas*. Commonly these pigments are referred to as fluorescens and generally have a greenish-yellow glow. The species *Ps. fluorescens* generates these types of pigments and is commonly associated with the spoilage of foods (eggs, cured meats, fish and milk). The FLOR-BART™ has been designed to generate these pigments where there is a dominance of fluorescent Pseudomonads (hence the prefix, FLOR). If *Ps. aeruginosa* is detected in a water sample and there is a concern for the potential hygiene risk, it is recommended that confirmatory diagnosis be performed in a recognized diagnostic microbiology laboratory using either the positive FLOR-BART™ or a fresh sample as the source for the diagnosis.

Other pigments are sometimes produced. These are usually insoluble and non-fluorescent in UV light. These are commonly yellow, beige or orange in color and tend to be transitory. One species *Ps. stutzeri* sometimes generates a reddish-brown pigment later in the growth cycle that is very distinctive. This pigment may concentrate either in the slime ring around the FID ball or in the base of the test vial.

Microorganisms present around the ball in the FLOR-BART™ can generate these different pigments in the presence of oxygen. Usually these pigments are generated after a cloudy growth has developed in the liquid medium but before there are intense slime-like growths around the ball (as a slime ring). The fluorescent pigments may be difficult to observe with natural and artificial light but they can be seen using a typical broad spectrum UV light whereupon the pigments glow (fluoresce).

There is often a need to test environments for the presence of Pseudomonad bacteria because these bacteria are often dominant in fluids which contain oxygen and are rich in a narrow range of organic pollutants (e.g., gasoline, jet fuel, solvents). When these bacteria are present and active, there are two particular events that may need to be considered. First, the presence of Pseudomonad

bacteria may indicate that aerobic biodegradation is occurring and biofouling may also be happening within the system being tested. Second, some of the Pseudomonad bacteria that produce the fluorescent (glowing in UV light) pigments may be a hygiene risk. The faster that clouding and fluorescing happens, the more aggressive are the Pseudomonad bacteria.

For the FLOR-BART™ there are two UV fluorescent pigments which can be recognized as:

**PALE BLUISH GLOW** that will last for one to four days and then gradually fade. The glow is normally fairly faint and should be viewed against a darkened background since direct light may make viewing more difficult. One major species bearing this pigment (pyocyanin) is *Pseudomonas aeruginosa*. It is of concern since this species can be associated with a range of opportunistic infections. It is also one of the bacterial species found associated with mastitis in cattle. This species can also be found in a variety of waters.

**GREENISH-YELLOW GLOW** that may last for two to ten days and then gradually fades away. The glow becomes fairly obvious and is often visible even without using the UV light. One major species bearing this pigment (the pyoverdin, fluorescein) is *Pseudomonas fluorescens*. Generally this species is not as virulent as *Ps. aeruginosa* and is often more abundant in waters and can be involved in specialized aerobic degradation of organic pollutants.

In essence, this test selectively allows the detection of Pseudomonad bacteria in the water with the separation of the fluorescent species. Pseudomonad bacteria can cause a range of problems in waters. Problems range through slime formations, turbidity, taste and odor, corrosion and biodegradation through to greater hygiene risks. In recreational waters (such as swimming pools, hot tubs, restricted natural bathing sites), the presence of aggressive fluorescent pseudomonads should be taken as a potential cause for concern since these bacteria may cause a range of skin, eye, ear and urinary tract infections. Occasionally the pseudomonad bacteria will cause skin infections particularly under tight fitting bathing apparel. This is particularly a potential problem in warmer waters and hot tubs where the bathers remain relatively inactive in the waters for prolonged periods.

The pseudomonad bacteria often dominate aerobic biodegradation of organic pollutants and determining the aggressivity and possible population size can often monitor the rates of degradation. If the organic pollutant is being degraded aerobically or in a situation where there is a significant quantity of nitrates to support respiration, there is a potential for the degradation to be dominated by the Pseudomonad bacteria. Monitoring the aggressivity of these bacteria using the FLOR-BART™ enables the user to monitor the amount of biodegradation occurring.

Pseudomonad bacteria are also sometimes associated with taste and odor problems in water since many of the species produce distinctive odors such as a "fishy" or a "kerosene-like" which can become very dominant in the water.

## 6.1 Reaction Pattern, Fluorescing Pseudomonads

| | | |
|---|---|---|
| **PB** | - | Pale Blue Glow in UV Light |
| **GY** | - | Greenish-Yellow Glow in UV Light |

These Reactions are described in more detail. Care should be taken to follow manufacturers cautionary notices when using an UV light source to observe glowing in the BART™ tests.

**PB – Pale Blue Glow**

Solution very cloudy and then generates a glowing around FID when ultra violet light is shone onto the side walls of the inner test vial. This glowing fluorescence occurs usually in the top 15 to 20 mm around the ball and gives a pale blue glow. This glowing commonly lasts 2 to 3 days

**GY – Greenish Yellow Glow**
Solution very cloudy and then generates a glowing around FID when ultra violet light is shone onto the side walls of the inner test vial. This glowing fluorescence occurs usually in the top 15 to 20 mm around the ball and gives a greenish-yellow glow. This glowing lasts commonly for 4 to 8 days (latter case).

## 6.2 RPS (Reaction Pattern Signatures) for the FLOR-BART™

- **PB** *Pseudomonas aeruginosa* likely to be present
- **GY** *Pseudomonas fluorescens* species group likely to be present

## 6.3 Time Lag (days of delay) to FLOR-BART™ Populations

**Table Eleven**

**The Relationship between Time Lag and the Population For Fluorescing Pseudomonad Bacteria**

| Time Lag (days) | Population cfu/ml |
|---|---|
| 1 | 1,000,000 |
| 2 | 100,000 |
| 3 | 10,000 |
| 4 | 5,000 |
| 5 | 1,000 |
| 6 | 100 |
| 7 | 100 |
| 8 | 100 |

The fluorescing Pseudomonads are only a part of typical slime forming or heterotrophic aerobic bacterial consortia. As such, they have to be in high populations and very aggressive in order to begin to produce the Ultra Violet (UV) fluorescence that is typical for the species of *Pseudomonas* that are capable of doing this. A time lag of longer than five days may have a small population but if the UV glow produced is pale blue. This would still be a concern if the nosocomial pathogenic bacterial species *Pseudomonas aeruginosa* was present in the sample and confirmatory tests using the traditional microbiological procedures may need to be undertaken as a precaution.

## 6.4 Risk Potential Assessment – FLOR-BART™

### 6.4.1 BART™ Extinction Dilution Technique

To measure the population of fluorescing pseudomonad bacteria, four dilutions of the original sample should be used. These dilutions can be achieved using the following semi-quantitative technique:

1. Dispense 14 ml of sterile water into each of four FLOR-BART™ tests. Label these tubes: “1”, “2”, “3”, and “4’.
2. Charge a FLOR-BART™ with the water sample (15 ml) and label “0”
3. Withdraw 1 ml of water from tube “0” and transfer into tube “1”. Invert and gently shake tube for 10 seconds. Allow to settle (5 seconds).

4. Withdraw 1 ml of water from tube "1" and transfer into tube "2". Invert and gently shake tube for 10 seconds. Allow to settle (5 seconds).
5. Withdraw 1 ml of water from tube "2" and transfer into tube "3". Invert and gently shake tube for 10 seconds. Allow to settle (5 seconds).
6. Withdraw 1 ml of water from tube "3" and transfer into tube "4". Invert and gently shake tube for 10 seconds. Allow to settle (5 seconds).
7. Observe the tubes for PB or GY fluorescence after three days of incubation at room temperature. Note that this day may be changed if an alternate day is found to display maximum fluorescence.
8. Refer to table below to semi-quantitatively determine population based upon the tests that exhibit fluorescence in an UV light.

**Table Twelve**

**The Relationship of Positive Detection of Fluorescence to The Population**

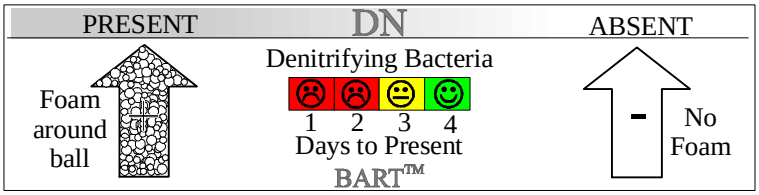
| Tube # | Population Assessment | | | | |
|---|---|---|---|---|---|
| "0" | F | F | F | F | F |
| "1" | F | F | F | F | ---- |
| "2" | F | F | F | ---- | ---- |
| "3" | F | F | ---- | ---- | ---- |
| "4" | F | ---- | ---- | ---- | ---- |
| Possible Population: | >5.0 | >4.0 | >3.0 | >2.0 | >1.0 |

(Log Fluorescing Pseudomonads/ml)

## 6.5 Hygiene Risk Considerations

If a PB reaction is observed, there is a risk that *Pseudomonas aeruginosa* may be present in the water sample and could cause an infection in humans. These infections can range from pneumonia to skin, eye and ear infections. Where a population is detected and confirmed using the extinction dilution technique described above, the tube "0" FLOR-BART™ should be submitted to a suitable microbiology laboratory to confirm the diagnosis. If the fluorescence is of the GY type, then a similar precaution should be taken if the population is >2.0 log Fluorescing Pseudomonads/ml.

## 7.0 DENITRIFYING BACTERIA, DN-BART™




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 the 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 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.

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:

$$\underset{\text{Nitrate}}{NO_3^-} \xrightarrow{(1)} \underset{\text{Nitrite}}{NO_2^-} \xrightarrow{(2)} \underset{\text{Nitric oxide}}{NO} \xrightarrow{(3)} \underset{\text{Nitrous oxide}}{N_2O} \xrightarrow{(4)} \underset{\text{Nitrogen}}{N_{2\,gas}}$$

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.

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* | *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$^{TM}$) 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.

## 7.1 Reaction Patterns, Denitrifying Bacteria

**FO** - Foam around Ball

Solution usually cloudy but the major positive for FO is the presence of very many bubbles collecting over >50% of the area under and around the ball to form a foam around the ball. This shows that complete denitrification has occurred and the denitrifying bacteria are present.
There is only one reaction recognized in the DN – BART™ that occurs when the nitrate is completely denitrified to nitrogen 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.

## 7.2 Time Lag (days of delay) to DN-BART™ Populations

Populations can be assessed by the time lag to the foam formation (Table Fifteen)

**Table Thirteen**

**The Relationship between Time Lag and the Population For Denitrifying Bacteria**

| Time Lag (days) | Population cfu/ml |
|---|---|
| 1 | 1,000,000 |
| 2 | 200,000 |
| 3 | 50,000 |
| 4 | 10,000 |

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.

## 7.3 Risk Potential Assessment –DN-BART™

### 7.3.1 BART™ Extinction Dilution

To quantify the numbers of denitrifying bacteria in the sample, a dilution (extinction) technique would need to be used. To measure the population of denitrifying bacteria, four tenfold dilutions of the original water sample should be used. These dilutions can be achieved using the following technique:

1. Dispense 14 ml of sterile water into each of four DN-BART™ tests. Label these tubes: “1”, “2”, “3”, and “4’.
2. Charge a DN-BART™ with the water sample (15 ml) and label “0”
3. Withdraw 1 ml of water from tube “0” and transfer into tube “1”. Invert and gently shake tube for 10 seconds. Allow to settle (5 seconds).
4. Withdraw 1 ml of water from tube “1” and transfer into tube “2”. Invert and gently shake tube for 10 seconds. Allow to settle (5 seconds).
5. Withdraw 1 ml of water from tube “2” and transfer into tube “3”. Invert and gently shake tube for 10 seconds. Allow to settle (5 seconds).
6. Withdraw 1 ml of water from tube “3” and transfer into tube “4”. Invert and gently shake tube for 10 seconds. Allow to settle (5 seconds).
7. Observe the tubes for **FO** (foam) after two days of incubation at room temperature.
8. Refer to Table Sixteen below to determine population.

**Table Fourteen**

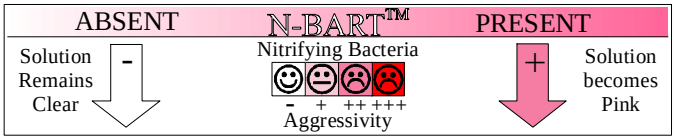
**Interpretation of the BART™ Extinction Dilution For Denitrifying Bacteria**

| Tube # | Population Assessment | | | | |
|---|---|---|---|---|---|
| "0" | FO | FO | FO | FO | FO |
| "1" | FO | FO | FO | FO | ---- |
| "2" | FO | FO | FO | ---- | ---- |
| "3" | FO | FO | ---- | ---- | ---- |
| "4" | FO | ---- | ---- | ---- | ---- |
| Possible Population: (log DN/ml) | >5.0 | >4.0 | >3.0 | >2.0 | >1.0 |

## 7.4 Hygiene Risk Considerations

Denitrifying bacteria flourish in environments 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 >3.0 log DN/ml, a coliform test should routinely be used to determine the health risk.

## 8.0 NITRIFYING BACTERIA, N-BART™




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

(1) (2)
$NH_4^+$ ----------→$NO_2^-$ ----------→$NO_3^-$
Ammonium Nitrite Nitrate

Nitrifying bacteria are divided according to which of the above reactions 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 groups are either producing or utilizing nitrate respectively. In developing a biodetection 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™ 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 is taken to indicate that there is a potential for significant amounts of nitrate to be generated in 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 a very significant and useful function 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 aggressive 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 reverse sequence of each other. 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.

## 8.1 Reaction Patterns, Nitrifying Bacteria

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. 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.

**PP** -Pink-red color on roughly half the ball
**RP** -All balls are reddened, solution may be pale pink
**DR** -Balls and the solution is reddened

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 FID hemispheres 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 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 ball. High concentrations of nitrite have been detected indicating an aggressive level of nitrification has occurred in the test period.

## 8.2 RPS (Reaction Pattern Signatures) for the N-BART™

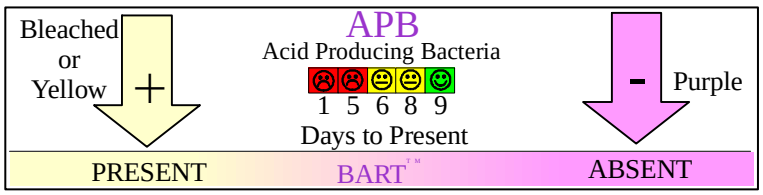
The reaction represents the population size and does not reflect the variety of microorganisms present in the water sample:

- **PB** Small population of nitrifiers ($< 10^2$ nitrifiers/ml) associated with aerobic slime forming bacteria in a consortium
- **RB** Moderate population of nitrifiers ($> 10^2$ and $< 10^5$ nitrifiers/ml) forming a major component in the bacterial flora
- **RT** Dominant population of nitrifiers ($> 10^5$ nitrifiers/ml)

## 8.3 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. 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.

## 9.0 ACID PRODUCING BACTERIA, APB-BART™



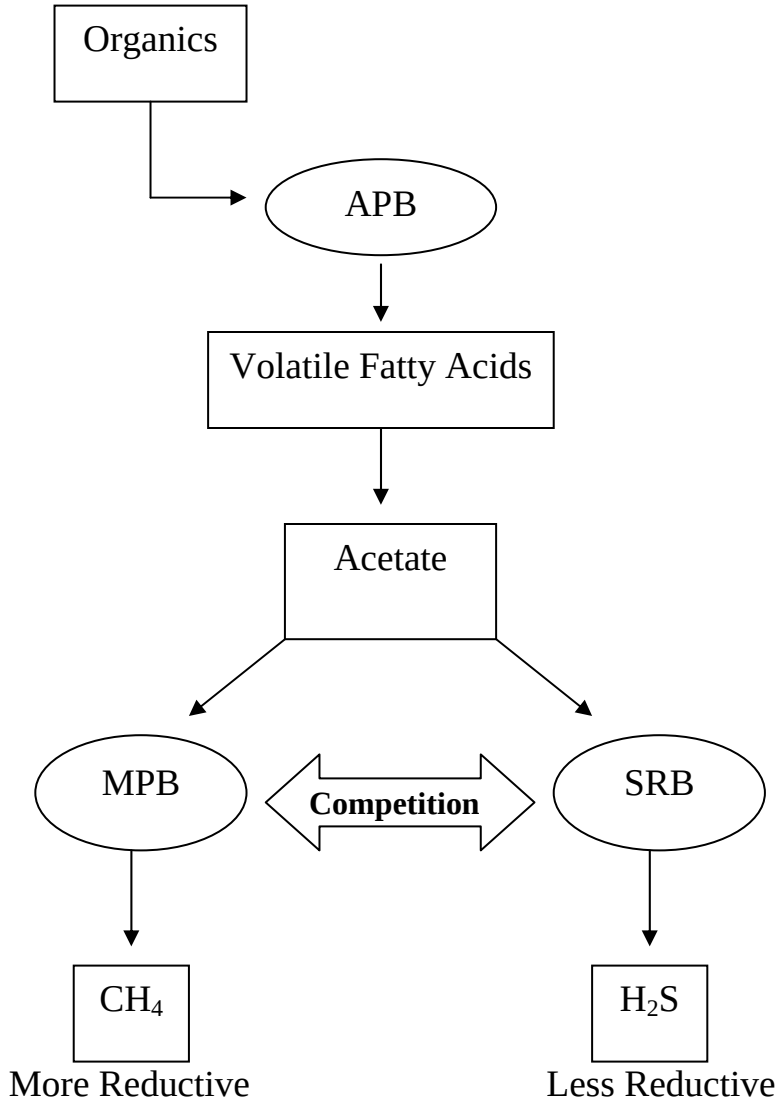

A major concern in the management of water systems, as well as oil and gas systems, is the risk of corrosive fluids. In the oil and gas industry, corrosion is a major concern because of the irreversible nature of the damages and the high cost of prevention (e.g., through cathodic protection and the application of biocides). Over the last forty years the cause of corrosion has shifted from being considered as a complex electro-chemical process to one that is commonly mediated by microorganisms. There are two groups of bacteria that can be instrumental in causing corrosion that include the sulfate reducing bacteria (SRB) and the acid producing bacteria (APB). This test focuses on the detection of acid producing bacteria as a potential cause of acidic waters and corrosion problems.

Historically, the recognition of microbially induced / initiated corrosion (MIC) placed emphasis on the SRB because of their clear links to the production of hydrogen sulfide which was well recognized as the initiator of electrolytic corrosion in steels. With the development of an understanding of the nature of biofilms in the production of slimes, tubercles, nodules and encrustations, it was found that there was a considerable amount of acidic products formed particularly deeper down in the biofilm. The association of some corrosion problems with the formation of acids led to the need to determine whether APB was involved. The acids produced by these bacteria are a result of fermentation of organics under very reductive (oxygen-free) environments. If oxygen were present, these APB would not be able to ferment significant amounts of acid. When fermented, the most common acids produced are the smaller fatty acids such as acetate, lactate, propionate and butyrate along with some of the volatile fatty acids.

The APB activity is a result of a community, or consortia, of bacteria rather than one single species. The exact nature of the bacterial species involved and the composition of the acidic products can vary throughout systems. It remains important that the APB can produce sufficient acidic products to significantly drop the pH at the interface between the biofilm and the supporting structures, and also in the water passing by the biofilm. APB is suspected when the pH in the impacted environment begins to fall into the acidic range under conditions when there is little or no oxygen available. If oxygen is present then the APB will not generate acid conditions in the water but may at the interface between the biofilm and the supporting material (e.g., concrete, steel). If acid production is occurring under oxidative conditions then the sulfur oxidizing bacteria such as *Thiobacillus* may be the cause. These bacteria can be detected using the AMD-BART™ commonly employed to determine the cause of acidic mine drainage.

Essentially, the APB could be viewed as setting up the conditions for increased levels of aggressivity by both the SRB and the methane (biogas) producing bacteria with both utilize the fatty acids generated by the APB. In this environment, there is microbial competition for the acetate (under reductive conditions) between the SRB and the bacteria able to generate methane. This latter group is known as the methanogenic bacteria (MPB) and can generate significant quantities of biogas. In the scheme of “things”, the APB ferment organics to acetate that is now

the prime substrate for SRB and MPB with the latter tending to dominate under the more reductive conditions.




The APB have now been recognized as a possible major cause of corrosion mainly because their fermentative activities will cause the pH particularly in the biofilms to drop into the acid range. Under these conditions, an acid-driven form of corrosion could occur, where the metals begin to dissolve and concrete structures lose integrity. This form of acid-corrosion can be viewed as an initiating, or alternating, event to SRB-initiated electrolytic corrosion. In the last two decades, industry has become more aware of the risks posed by the APB and have come to generally view the creation of acidic pH levels within the environment under reductive conditions to be predominantly driven by the APB. This heightens the corrosion risk to the engineered systems within the affected zone. To detect the APB, an APB-BART™ has been developed.

One of the major problems in establishing the APB-BART™ test has been to determine what level of acidic pH can be considered significant to increase the corrosion risk? A survey of the concern revealed a wide disparity in the pH values that would be considered threshold for an

increased acid-induced corrosion risk. In general the range was from as low as 3.8 to as high as 6.2 with most moderating in the range of 4.4 to 5.8. In the development of the APB-BART™ it was decided to work with a range of produced and natural waters as well as ATCC bacterial strains to set a threshold pH value to act as a positive detection of APB. Initially, threshold values were set in the pH range of 4.4 to 4.8 but this was found to generate a high probability of false negatives. Examination of the normal pH operating range for the APB to generate acidic products was found to be much higher than the 4.4 to 4.8 range and, by experiment, it was found that the threshold range that would be triggered by APB would be in the 5.2 to 5.8 pH range. When this was selected as the target range to confirm the presence of APB there appeared to be a greater conformity between detectable (by the APB-BART™) and confirmed risk.

The next challenge related to the speed of the APB-BART™ in detecting the presence of acid producing bacteria in the water samples and pure cultures. It was found that, in the earlier configurations of the test, there was a slow and dispersed acidic generation that was resolved by modifications to the supporting culture medium. This medium can be seen as a crystallized deposit on the floor of the inner BART™ test vial. The approved medium causes acid formation along much of the water / medium column in the test and a positive reaction is now easy to determine by the clear shift in the color of the solution from purple to yellow during the incubation of the test at room temperature.

Comparative studies of the APB-BART™ against the standard American Petroleum Institute's standard test for acid producing bacteria conducted by an oil company in Alberta revealed that the APB-BART™ detected the APB faster in the water samples thought to have a problem than did the standard method. In addition, the APB-BART™ detected these bacteria being active in more samples than the standard method. It is recommended that in using the APB-BART™ comparisons be made with the standard methods and that some of the suspension from a positive BART™ test be applied to the standard test to confirm that the APB detected as present are confirmed as acid producing bacteria.

The medium selected for the detection of the APB is a glucose-peptone-based medium incorporating a pH indicator, bromocresol purple. This pH indicator shifts from a purple color under neutral to alkaline conditions to yellow under acid with the transition occurring between pH values of 5.2 and 5.8. While the medium is in the form of a crystalline pellet on the floor of the inner BART™ test vial, the pH indicator is impregnated into the inside of the cap. The test is initiated by adding the water sample (see notes 1 and 2 for constraints and limitations) to the inner test vial until the water reaches the fill line with the ball floating up to the surface. To charge the test, the inner test vial when sealed with the cap is inverted for thirty seconds to allow the pH indicator to mix with the water. This starts the test and the water sample in the test should have a purple color (see note 1 for more information if the water is either a yellow or golden orange color). It is recommended that the test be read daily for any significant change in color while being kept at room temperature away from direct sunlight.

## 9.1 Reaction Patterns, Acid Producing Bacteria

A positive reaction is indicated by a change of color within the test vial. The color of the fluid when the test is initiated is a deep purple. When the test has gone positive the purple color will have changed to an orange or yellow. This color change should be cloudy or followed by clouding within the test vial. Normally this reaction will appear at first in one or more zones down the test vial and these will gradually spread until more than 80% of the sample has turned from a purple to a yellow-orange color. Note the first time that this color change is occurring and calculate the number of days that the test was running before the positive indication of acid production (yellow-orange color) was noticed. This time, usually measured in days, becomes the time lag (TL) from which the aggressivity of the APB can be calculated.

## 9.2 Time Lag (days of delay) to APB-BART™ Populations

There is only one reaction recognized in this test. This reaction is indicated when produced acid (PA) results in a change of color in the sample being tested. The TL gives a measure of the aggressivity of the test:

- TL of 3 days and less - HIGH AGGRESSIVITY
- TL of 4 to 6 days inclusive - MEDIUM AGGRESSIVITY
- TL of 7 to 10 days inclusive - LOW AGGRESSIVITY

TL can also be used to determine the population of APB using Table Seventeen.

**Table Fifteen**

**The Relationship between Time Lag and the Population For Acid Producing Bacteria**

| Time Lag | Population cfu/ml |
|---|---|
| 1 | 1,000,000 |
| 2 | 500,000 |
| 3 | 100,000 |
| 4 | 50,000 |
| 5 | 10,000 |
| 6 | 1,000 |
| 7 | 100 |

## 9.3 Hygiene Risk Considerations

Some of the APB belongs to the section five bacteria. Included in this section are the enteric bacteria within which the coliform bacteria form an important part. While the reductive conditions found in an environment generating acidic environments would not be likely to support the growth of the principal coliform species, *Escherichia coli*, some of the other species associated with coliform bacteria and hygiene risk could be present. In the event of a highly or moderately aggressive APB population being detected (with a TL<7days) it is recommended that a total coliform test be conducted on the sample to determine the hygiene risk with respect to coliform bacteria.

### 9.3.1 Notes on Constraints and Concerns

### Acidic Water Samples: Modification to the Testing Procedure.

Water samples that are acidic (i.e., have a pH<6.0) are likely to give an instant or premature positive reaction (PA). It is therefore necessary to correct the sample's pH by titrating the pH upward with sterile normal KOH. This will raise the pH up into the range of 6.9 to 7.2. This does create some trauma when the test is then performed. This is usually because the APB becomes stressed by the sudden upward shift in pH resulting from the addition of the KOH. It is therefore recommended that the TL obtained by this technique would need to be modified to correct for the trauma. This is done using the following formula:

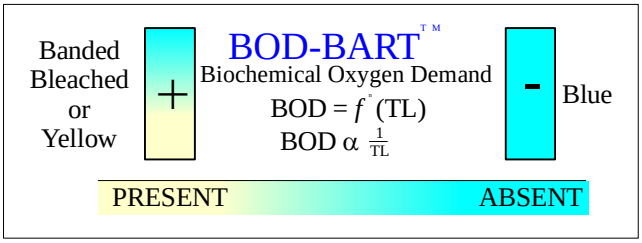
$$TL_c = TL_r - 2$$

Where $TL_r$ is the time lag actually observed for the sample in which the pH was amended using the sterile KOH and $TL_c$ is the corrected TL that should be used to determine the aggressivity and population size.

## High Saline Waters: Modifications to the Testing Procedure

Water samples containing greater than 6% salt is most likely to give false negatives. The high concentration of salt within these samples interacts negatively within the test vial, giving a negative reaction. This is not a definitive negative however, to rectify this problem, all samples over 6% salt concentration should be diluted. To determine the presence of APB in such waters it is recommended that the water sample be diluted with sufficient sterile distilled water to bring the salt concentration down to less than 6% before setting up the APB-BART™ test. The dilution of these types of samples to < 6% gives a much improved potential to detect the APB in highly saline waters (e.g., connate / produced waters).

## 10.0 BIOCHEMICAL OXYGEN DEMAND, BOD-BART™




*TL Time Lag/Time to Positive Reaction

Biochemical Oxygen Demand (BOD) is the measure of oxygen consumption in water bodies due to the biological oxidation of organic matter. Presently, BOD is measured by using a five-day standard test based on dilution techniques. In comparison, the BOD-BART™ test provides an easy and rapid (< 24 hour) measurement of BOD based on enhanced respiration activity of heterotrophic aerobic bacteria. Accurate determination of the respiration rate of HAB (i.e. the rate of free oxygen uptake) in terms of time to positive reaction or time lag (TL) is the essence of the rapid determination of BOD or the concentration of biodegradable organic matter within a sample. Heterotrophic aerobic bacteria are able to biodegrade or consume organic matter in water bodies as their source of energy, using free available oxygen as an electron acceptor. By far, the majority of these heterotrophs function most effectively under aerobic conditions. Much of the biodegradation that occurs in aerobic environment is due to the activities of HAB. Since these bacteria are primarily responsible for oxygen demand in water bodies, their respiratory activity has serious implications on the quality of water within these bodies.

The unique feature of the BOD-BART™ test is the addition of a specific mineral and nutrient media and methylene blue, as a redox indicator. These additives enhance the respiration rate and its measurement as a function of time lag through time to initiation of bleaching (blue color changes to clear state). While there remains free oxygen in the sample, the methylene blue dye in the liquid medium remains blue. As soon as all of the oxygen has been consumed by bacterial respiratory activity, the methylene blue shifts from its observable form to a colorless form. In other words, in the BOD-BART™ test, when the liquid medium turns from blue to a colorless form, the heterotrophic aerobic bacteria have been sufficiently aggressive to have “respired off” the oxygen. At this time a methylene blue reductase enzyme becomes active and this starts reducing the methylene blue to its colorless form. Here the rate of bleaching action is correlated with the concentration of BOD in the sample. Note that the dried methylene blue present in the cap of each biodetector is dissolved in the liquid by inverting the BOD-BART™ three times up side down. During this process the FID traverses up and down the test vial six times. This allows for a head space oxygen saturation in the liquid.

Methylene blue is a basic dye that can bind readily to the negatively charged microbial cells. Traditionally, this dye has been used to stain microbial cells. The important property of methylene blue dye is that it changes from its original blue color in the oxidized state, to a clear in the reduced state with a progression of oxygen consumption. When methylene blue is added to a liquid medium with biodegradable organic concentration, due to microbial respiration, electrons are transformed to the dye carrying it to become reduced and eventually the blue color starts disappearing, depending upon rate of biological respiratory activity. The protocol has been based

on the methylene blue reductase test that has been used in dairy industry for decades to determine the potential for bacterial spoilage of milk.

In the BOD-BART™ main objective is to provide an easy and rapid alternative system for determination of BOD in wastewater's as BOD is considered to be an important parameter for water pollution control activities.

## 10.1 Reaction Patterns, Biochemical Oxygen Demand

**UP** -Bleaching moves upwards from base

There is only one recognized reaction pattern (UP) for the BOD-BART™. There are different forms of clouding which follow the bleaching of methylene blue and these can be recognized using raw data from the BODSCAN™ system, although it is not important for BOD determination point of view.

**UP- Bleaching moves upwards**
Blue solution bleaches from the bottom up. The bleached zone may be clear or clouded. In the latter case, the medium tends to have a light to medium yellow color. Rarely does the bleaching extend beyond the equator of the ball so that a blue ring will remain around the ball with a width of 1 to 5 mm.

## 10.2 RPS (Reaction Pattern Signatures) for the BOD-BART™

- **UP** Strict aerobic bacteria is dominant with some facultative anaerobes often present

## 10.3 Time Lag (hours per second) to BOD-BART™ Concentration

The relationship between the approximately time lag (hours per second) to the probable BOD (mg/L) is given in Table Eighteen.

**Table Sixteen**

**The Relationship between Time Lag and the Probable BOD**

| Time Lag (Hours) | Probable BOD (mg/L) | Time Lag (Hours) | Probable BOD (mg/L) |
|---|---|---|---|
| 1.0 | 667 | 8.0 | 31 |
| 1.5 | 368 | 9.0 | 26 |
| 2.0 | 241 | 10.0 | 23 |
| 2.5 | 174 | 11.0 | 20 |
| 3.0 | 133 | 12.0 | 17 |
| 3.5 | 106 | 13.0 | 15 |
| 4.0 | 87 | 14.0 | 14 |
| 4.5 | 73 | 16.0 | 11 |
| 5.0 | 63 | 18.0 | 10 |
| 5.5 | 55 | 20.0 | 8 |
| 6.0 | 48 | 24.0 | 6 |
| 7.0 | 38 | >24 | Not Detected |

## 10.4 Risk Potential Assessment-BOD-BART™

The BOD-BART is specifically designed to determine the biochemical oxygen demand within a sample. This BOD number determines the risk associated to the environment, in particular, water bodies. Regular and rapid monitoring of BOD data is critical to control pollution loads in receiving water bodies. BOD has serious implications on environmental quality of these water bodies. If not controlled properly, it adversely impacts the health and quality of aquatic systems, in turn, posing a health risk to humans. This is a result of reduced levels of dissolved oxygen in water bodies. This remains a demand for a rapid BOD forecasting system to cope with this type of environmental risk without delays. Besides rapid evaluation, BOD is also important in controlling unit operations in wastewater treatment plants. BOD-BART™ with BODSCAN™ would provide an attractive solution to above concerns and help in minimizing the pollution risks in water bodies.