Chapter 3

Bart Tester Definitions

3.1 Introduction

One of the principal factors affecting the success of the testers over the last twenty five years has been the relatively simple methods that the operator can use to detect selected bacterial activity. These activities range from corrosion of pipes, plugging in the lines, discolouration in the water, odors, to causing general system failures. Today these types of bacteria are commonly referred to as `nuisance bacteria` and have as much importance as the pathogenic (disease causing) bacteria. In large part this is achieved by the inclusion of the correct selective chemical nutrient pellet that would, upon diffusion into the sample column of the tester, trigger the activities and growth of only those bacterial communities that are the subject of that specific investigation.

This Chapter addresses the form of the proprietary chemical nutrient pellet and the weight incorporated into each of the different lsb (inner) Bart testers. Included is the definition of the proprietary formula involved, the weight of the pellet when dried following the standard ISO 9001: 2000 protocols, and a brief definition of the of the bacterial types that can be detected. All pelleted media are dried to a constant weight at 52 ± 2^{o}C with drying times standardized to eight days. Each tester type employs one (laboratory version) or two (field version) caps that are colored in a distinctive manner to define the type of tester. Colors where relevant to the interpretation of any given tester reaction are described incorporating the Xerox Pantone® colors defined on July 25th 2007 with all rights reserved. Standard colors employed were printed on the Phaser® printer (8560DN) using the standard range of Pantone® colors.

3. 2. Iron Related Bacteria (IRB- BART)

– Bright Red Cap

Parameter code: iron biotester

IRB have a unique feature in that it participates in some part of the cycling of iron in the natural environment primarily as iron oxidizing (generating insoluble ferric forms of iron) and iron reducing (generating soluble forms of ferrous iron). Traditionally bacteria that are reductive generating ferrous forms of iron are called iron reducing bacteria` and bacteria that function oxidatively generating ferric forms of iron are referred to as the iron oxidizing bacteria`. Some bacteria can perform both of these functions and so the definition of iron related bacteria is applied (Cullimore, 2008 and 201-0) to:

> "incorporates all bacteria that are able to accumulate beyond basic metabolic needs iron in any form within the environmental matrix where they actively function"

Selective chemical nutrient pellet is based on ferric ammonium citrate with other essential nutrients for IRB activities. It has been found that there is an intrinsic reduction of the ferric-iron to the soluble ferrous form in the reductive base of the charged tester if IRB are present and active. If iron reducing bacteria dominate in the sample then there will be a dark green color in the lower (reductive) region generated over the diffusing basal pellet and, if these bacteria dominate, then the whole column may eventually turn a lighter shade of green. In the event that iron oxidizing bacteria dominate then the color being generated above the diffusing basal pellet is commonly yellow but occasionally this will have an orange tinge. In both cases the sample column will remain relatively clear (free from clouded growths) and this is not used the prime differentiation in this tester (see also 3.1.2.1.). IRB- pellet dried has averaged weights per tester of 349.5±1.5mg

3.2.1 Reaction patterns for the IRB- Bart

Of all of the BART testers it is the IRB-Bart that generates the most reactions and there are four potential phases of reactions that can occur in the IRB- tester. These four phases make the

determination of specific bacteria much more challenging than for other testers. The first phase that sometimes occurs is not used in the identification of the types of iron related bacteria but does indicate some characteristics of the sample that should be noted. The next two phases (2nd and 3rd) are recognized as relating to particular IRB activities. Phase two simply differentiates the IRB activities into oxidative (respiratory) or reductive (fermentative) types. Generally it is the phase two reactions that trigger the first reaction that is used to determine time lapse (and hence the predicted active cell population for that sample). Phase three now recognizes the subsequent reactions (3rd phase) that occur in sequence during the incubation of the tester after the phase two populations has been determined. Phase four is a terminating event (black liquid, BL) after which no further reactions will occur and the test may be considered to be finished.

3.2.1.1 Phase One IRB Reactions

Phase one reactions are not used in the identification of IRB as such but should be recorded and can provide some insight into the types of environmental conditions that have been triggered by the interaction between sample and the tester. These reactions are discussed below:

- **White base (WB)** as a reaction occurs in the conical base of around 70% of the IRB-testers forming at between 30minutes and 10hours into the incubation period. This reaction is easy to recognize because the conical base of the tester turns white with crystalline deposit. VBR testing reveals the deposit building up does not occur as layers but is an event that occurs quite quickly (taking often less than one hour to complete). Analysis reveals that the dominant chemicals are carbonates. Volume occupied in the basal cone ranges around 1.2±0.2mL and once formed (as a white base, WB) it generally remains stable for the balance of testing period. In practice the occurrence of the WB reaction in phase one is indicative that the any treatment to remove biomass influencing biofouling should include acidic treatment in at least one phase to dissolve the carbonates and aid in the destabilization of the biomass.
- **Green diffusion (GD)** commonly occurs from the base of the tester upwards and is recognized as a dark green reaction (Pantone 360 to 362) at the base moving to a mid-green reaction ascending up to the BART ball. In this reaction the solution is clear (not cloudy). This reaction indicates that there is a reductive condition with ferrous forms of iron dominating. This would indicate that iron reducing bacteria are likely to be both

present and actively reducing the ferric-iron found in the selective chemical nutrient pellet and possibly in the sample to the ferrous form. While GD can be used as evidence of ferric-iron reducing bacterial activity it is used only as a confirmatory reaction in phase one.

- **Yellow diffusion (YD)** commonly occurs through the column of the tester at the same time. Generally this is a clear light yellow solution that may gradually shift on a few occasions to a shade of light orange (Pantone 7403 to 7405). This is an iron oxidizing reaction where the ferrous- and ferric- forms of iron are being mobilized as ferric-rich yellow colloidal material in the tester sample column. This is an early activity of the iron oxidizing bacteria but should not be treated as a positive detection unless a phase two reaction is observed.

Summary of these three phase one reactions is that the WB reaction may be used as an aid in the development of chemical treatment strategies since carbonates could be integrated into the biomass. WB cannot be used as diagnostic for specific IRB activities. GD and YD reactions can be used to consider that iron-reducing or iron-oxidizing activities are occurring respectively but they are of limited scope and require phase two confirmation.

3.2.1.2. Phase Two IRB Reactions

There are two reactions that occur in phase two that can used to define the type of IRB activity that is occurring in the sample. Phase two in essence generates the first recognized reaction. Commonly it is this (phase two) reaction that then triggers prediction of the population (as pac/mL) from the time lapse. These reactions are very recognizable and reflect fermentative or respiratory activities (as FO and CL respectively). On some occasions the BR (brown ring) reaction may occur before foaming or clouding and may, where this happens, be considered to be a phase two reaction. The major phase two reactions are described as:

Foam formation (FO) is recognized only when there is a complete foam ring of bubbles (or more) formed around the ball. These bubbles each contain gas which is restrained within biofilms (slime coatings). Common gases associated with foam formations are some combination of carbon dioxide, methane, nitrogen, and hydrogen. The manner in which the foam is generated is a reflection of the dominant gas within the foam. Nitrogen tends to be the most durable foam gas lasting for several days in the foam while methane is the least durable since it is rapidly degraded under the oxidative conditions that would exist under the headspace air. Foam dominated by carbon dioxide along with hydrogen generally collapse fairly quickly. Gas bubbles formed on the sides of the tester (usually in the early stages of incubation) are most probably oxygen that has been released from the super-saturation state in the liquid sample as temperatures has moved up to that of the room. FO indicates that fermentative activities are dominating in the tester with the evolution of gases that then become wrapped in biofilms to form the bubbles that then lock into the foam layers around the ball. If the foam forms and continues to grow then this is most likely nitrogen. If the foam forms quickly and rises rapidly but collapses then that is most likely to be methane based and it may trigger biomass activity around the ball. Carbon dioxide dominated foams tend to grow slowly and then collapse slowly without sign of great biomass activity. FO reaction therefore means that the sample is likely to be from a reductive origin with a dominance of fermentative IRB. It should be noted that the brown ring (BR) can sometimes form over the foam as a ferric-rich brown crust. There is no standard Pantone color for the foam but pixels within the foam zone would vary considerably in their quality from white (inside the gas bubble) to grey, orange or brown depending upon the degree of bio-oxidation occurring.

Cloudiness (CL) tends to be a common first reaction occurring in the IRB-BART tester where the sample has come from oxidative (respiratory) types of activities are occurring. It commonly arrises from phase one yellow diffusive (YD) reaction using the same Pantone numbers of 7403 to 7405. Cloudiness is recognized by e losses of clarity in the sample due to the presence of growing biocolloidal particles. These may be dispersive in their form or they might be seen as growing within specific zones in the tester. When CL occurs then it is most probable that the sample is oxidative and supporting respiratory functions that are first seen as a generalized clouding (turbidity) in the tester. This commonly precedes the phase three reactions (see Chapter 3.2.2.3. below).

3.2.1.3. Phase Three IRB Reactions

Phase three incorporates the bulk of the recognized reactions seen in an IRB- tester except FO and CL (3.2.2.2.) and BL (3.2.2.4) along with the phase one reactions WB, GD and YD described in 3.2.2.1. These phase three reactions can be grouped around whether they occur oxidatively or reductively which is again a reflection of the bacterial communities that are present in the sample. The reactions that are recognized include:

- **Brown Cloudy (BC)** occurs when the sample medium solution turns to a shade of brown (typically Pantone colors 729 to 730). Generally this reaction follows a BG or is coincident with the BR reactions and is a terminal reaction for phase three.
- **Basal Gel (BG)** occurs commonly in the lowest third of the IRB- tester. They are easily recognized since the biomass form a gel-like growth within that zone and may have smooth or irregular edges at the interface with the solution above. Commonly in a BG reaction the solution above remains relatively clear (not cloudy) and has no distinctive color. The BG itself may take on a number of colors that do vary somewhat in intensity. Brown BG reactions (Pantone 463, 4635, 464, and 4645) and generally last for one to three days before terminating in a BC reaction. Green BG reactions have also been observed where there is initially a green banding (Pantone 576) which sets up just above the cone of the tester followed by the gel-like biomass shifting from a dark green (Pantone 7496) to a greenish brown (Pantone 7497) before terminating as a BC when the basal gel dissipates.
- **Brown ring (BR)** commonly occurs very quickly (Pantone 7516 to 7517) above the equator of the ball with growth commonly occurring at the headspace air- liquid sample medium interface. Generally the BR is seen as a gelatinous colored ring that initially has a glossy coating. Once formed the BR is durable and will still be visible even when phase four BL has occurred. Conditions within the BR are oxidative (dominated by ferric forms of iron) and is frequently dominated by the very aerobic iron related bacteria can be observed microscopically (x40 or x100 using transmitted light) growing within the ring of growth around the ball. BR is particularly a favourable environment for the sheathed and ribbon-forming iron oxidizing bacteria such as *Gallionella*, *Sphaerotilus*, *Crenothrix*

and *Leptothrix*.

- **Green Cloudy (GC)** indicates the presence of soluble ferrous-iron (Pantone 360 to 364) but a reaction is only called when the sample being incubated becomes cloudy. Generally this reaction darkens over time to Pantone 364 and the growth is caused iron-reducing bacteria.
- **Red Cloudy (RC)** is a reaction which generally initiates as a bright red (Pantone 485) which gradually darkens (Pantone 484). Commonly this reaction occurs in the entire sample medium column and the growth is often dense due to the presence of slime forming bacteria. The red color is thought to be generated by heterotrophic iron oxidizing bacteria oxidizing iron to the ferric form but some bacteria may also generate red pigments to augment the color.

3.2.1.4. Phase Four IRB Reactions

Phase four reaction is terminal and can be recognized by the sample medium column in the tester becomes a Black Liquid (BL). Closer examination of the BL reaction reveals that the blackening is caused by jet black particulated deposits on the testers walls while the sample medium column itself becomes clear. Tilting the tester with light behind the tester commonly shows that the medium is actually clear (without color) while the blackening (Pantone Black 6) has a granular nature and is firmly attached to the walls of the tester. These blackened deposits may be either forms of iron sulfides or carbonates or reduced forms of organic carbon which have been stripped of the more useful nutritive elements (e.g. phosphorus, sulfur, nitrogen, potassium and oxygen). This is commonly a terminal bio-reductive event within the tester when it happens.

3.1.3 Bacterial consorms recognized by reaction signatures in the IRB- BART

Bacterial consorms as defined in Cullimore (2010) may generate some of the reaction patterns as defined above in Chapter 3.1.2. They are listed in Table 3.1.3 by consorm type and then the most probable sequence for the reactions. Reaction patterns are shown in this table for each of the defined bacterial consorms as rows with the probability of the reaction shown in the form of asterisks (*) with the interpretation being: *** for highly probable occurrences; ** likely to occur with a moderate probability; * observed on some occasions but relatively uncommon; no

asterisk means that this reaction does not occur. Sequencing of the reactions would be from phases 2 to 4 with the order of the reactions observed in the IRB- testers being of possible diagnostic significance. Communities included in the above table include ROT- rotting; SLM- slime forming; BPL- black plug layers; BWR- black water; CGG- clogging; CCR- concretion; FRD- ferric-iron rich deposit; IPN- ferric-iron pan; OCR- ochre; PGI- pig iron; and TCL- tubercle. All of these communities do incorporate IRB activities. Additional information can found on significance of the observed reaction patterns for the IRB-BART tester in Cullimore (1999) pages 160 to 165 and Cullimore (2008) from pages 219 to 224. Section 3.12 defined the major IRB communities by the Standard Bacteriological Community Code (BSCC) and in 3.13 the major Pantone colors are define. These sections also address the SBCC for all of the other Bart tester types.

Note that is Table 3.2.1 it shows the occurrences of different reactions observed during testing. Typical phase two reactions are shown in columns 2 and 3; phase three reactions in columns 4 to 7 and phase 4 is column 8. The likelihood of the reaction is expressed by the number of asterisks in the cell.

3.1.4 Time lapse determination of predictive active cell populations

While Chapter 3.1.2 primarily address reaction recognition and interpretation, the first reaction recognized (commonly a phase 2 reaction) which triggers measurement of the time lapse. Time lapse is defined as that length of time that occurs between the start of incubation of the test and the specific time at which the first reaction was recognized and recorded. Time lapse can be measured in days, hours or seconds and the interpretation then predicts active cell population. This can be determined using: (1) the certificate of analysis which accompanies each box of testers based on days; (2) the entry of the time lapse into QuickPop, VBR and CBR software all automatically calculates the population as predicted active cells per ml (pac/mL) which may be considered equivalent to the traditional colony forming units per ml (cfu/mL) based on a choice of seconds, minutes or hours; or (3) using the full time lapse video (VBR I or II) system that allows the digital storage of time lapsed images of the IRB- testers over the time that the tests were incubated.

Table 3.2.1 Probable IRB-BART tester reactions for selected bacterial consorms showing phase two, three, and four reactions.

Community	Phase 2		Phase 3					Phase 4
	CL	FO	BC	BG	BR	GC	RC	BL
2, 16-13 CLB	*	***	*	**			***	***
2, 15-18 CLW	***		**	**	**	**		*
2, 10-12 FEC		***	**	**			***	***
2, 09-05 MIC	*	***	*	*		*		
2, 17-15 PLG	***	*	***	**	***		**	*
2, 14-15 ROT	*		*			**	**	**
2, 21-22 SLM			**	**	**	**		
3, 10-21 BPL	**	**	***	**			**	***
3, 03-16 BWR		***	*	**			**	***
3, 05-27 CGG	***							
3, 18-25 CCR	***	*	*	*		**		
3, 18-25 FRD	***	**	***	**	***			
3, 22-18 IPN	***	*	**		***			
3, 18-19 OCR	**	**	***	***	***	**		
3, 10-30 PGI	***		**		***			
3, 15-17 TCL	**	**		***	***			**

Note: CLB refers to coliforms, CLW to slime formers, FEC refers to fecal coliforms, MIC relates to corrosion, PLG relates to plugging, ROT is rotting, SLM is slime formers, BPL is black plug layer, BWR is blackened water, CGG is clogging, CCR relates to the formation of concretions, FRD ferric rich iron deposits, IPN is iron panning, OCR relates to ochre formation, PGI is the formation of pig iron from ferric rich bio-concretions, and TCL relates to the communities generating tubercles.

VBR systems I and I are suitable for the IRB- tester using the floor illumination allows recognition of the various reactions. Software that comes with the VBR software that allows interpretation along with the saving of the data entered. The generation of the relationship between time lapses and the predicted population has been made more challenging by the fact that natural samples often have complex microbial communities with which IRB are integrated. At all times the microbial community that is detected is subject to changes with shifts in the micro-environments and the aging process within the biomass.

Relating the time lapse to predicted populations (as pac/mL) is always has challenges due to the ongoing on-going shifting of activities within the community. The approach to these challenges differs with the tester type. For the IRB- tester, blended approaches have been employed to generate a best fit analysis (for the reactions observed and time lapse achieved) that would then be used to generate an acceptable fit. To generate precision in these evaluations eight pure bacterial cultures (Table 3.1.4.1) and three natural samples are routinely employed. Note that for some of these cultures, tests will shift from one reaction type to another as the growth matures. For example, *Citrobacter freundii* may cause after 5 to 8 days a bio locking of the ball so that when the tester is turned upside down the ball remains "glued" into position with the liquid medium held above the ball.

For the nine ATCC bacterial species the links were determined by culturing these on quarter strength brain-heart infusion agar, washing of the colonies off after three days of incubation at 30 ± 1^{o}C (except *E. coli* which was incubated at 37 ± 1^{o}C) using sterile ringers (saline) solution. These concentrated cell suspensions are then subjected to tenfold dilution series using Ringers down to 10^{-9}. Each dilution was then inoculated into an IRB- tester (15mL suspension) and incubated at room temperature (22 ± 2^{o}C) and observed daily for the generation of the reaction as specified in table 3.2.2 Concurrently populations in the original cell suspension are enumerated using dilutions and quarter strength brain-heart infusion agar spread plate colony enumeration with thirty to three hundred countable colonies being acceptable for calculation of the cell populations.

Table 3.2.2 IRB pure cultures used to project time lapse to population linkages

ATCC	Genus/species	Dominant Reaction
8090	*Citrobacter freundii*	GC
13048	*Enterobacter aerogenes*	BR
27853	*Pseudomonas aeruginosa*	GC
19606	*Acinetobacter calcoaceticus*	GC
23355	*Enterobacter cloacae*	CL-BG
13315	*Proteus vulgaris*	CL-BC
13883	*Klebsiella pneumoniae*	RC-BC
8100	Serratia marcescens	BR*
25922	*Escherichia coli*	FO

Note: *preferred quality management test giving a consistent brown ring reaction commonly by the fourth day.

For the three natural samples from ochre, 3, 18-19 OCR; and two ferric-rich plugs, 3, 19-21 PLG were used when extracted from the oxidative side of the ground water biomass. All three samples were examined and found to contain sheathed and ribbon forming iron related bacteria along with other iron oxidizing bacteria. These three samples were porous solid samples which were crushed in a sterile mortar and pestle to a fine grained powder. 1.5g of the powder was then dispensed into a sterile IRB- tester (without media and ball). After mixing with sterile distilled water (SDW) for thirty seconds 15ml was removed and dispensed into the regular IRB-laboratory tester. A serial dilution was now made using tenfold dilution with 1.5ml transfers made up with 13.5ml of SDW into fresh sterile IRB- laboratory testers followed by thirty seconds of mixing. Dilutions continued down to 10^{-7}. Incubation was at room temperature and the day that the first reaction occurred was used generate the time lapse relationship to the original population of IRB in the sample. Using data obtained by these two methods a "best fit" was generated which related the population sizes of both the ATCC pure culture strains and the three natural samples to time lapse. Regression analysis of the data found that equation one gave the most suitable correlation:

$$y = -0.6062x + 6.361 \text{ (equation one)}$$

In equation one x is expressed as the time lapse in days and y is the predicted population expressed as pac/mL the log base 10 in Table 3.2.3

Table 3.2.3 Daily Observations of the IRB-BART tester showing variability in Predicted Populations (pac/mL)

Note:

	Time Lapse (days			
	1	**2**	**3**	**4**
High population	1,990,000,000	494,000	122,000	30,000
Average population	**598,000,000**	**140,000**	**34,800**	**8,630**
Low population	161,000,000	40,000	9,900	2,450

	5	**6**	**7**	**8**
High population	7,500	1,850	460	114
Average population	**2,100**	**529**	**131**	**32**
Low population	608	150	37	9

Populations are expressed as predicted active cells per mL (pac/mL) based upon daily observations being inputted into QuickPop software with the time lapses are based on days above. For each of the eight days of observation the average population is that generated by QuickPop with the high and low population range created as a range from one hour after the previous daily reading to twenty three hours after the observation. For example on day two the predicted population would be 140,000pac/ml but the semi-quantitative range would be from 494,000 down to 40,000pac/ml. Quantitative information can be generated by more frequent observations using VBR I or II systems which can achieve acceptable precision. Intervals commonly employed for the VBR generation of time lapse data is most commonly every 15minutes.

3.3 Sulfate Reducing Bacteria (SRB-BART)

– Black Cap

Parameter code: sulfide biotester

3.3.1 Introduction

Sulfate reducing bacteria (SRB) are a group of anaerobic bacteria that, as a part of their normal activities, generate hydrogen sulfide (H_2S). This group (SRB) can be more precisely considered as the "sulfide producing bacteria" and as such are believed to be the most significant group recognized in corrosion processes. H_2S can cause a number of significant problems in water. These range from "rotten egg" odors, through to the blackening of equipment, waters and slime formations, and the initiation of pitting and perforation in corrosive processes. Detection of these microorganisms is made the more challenging because they are anaerobic and tend to grow deep within the biomass as a part of the microbial community. Detection of the SRB is therefore made even more difficult because SRB may not be present in the free-flowing waters over the site of the corrosion but are growing deeper down within the biomass. Because of this, symptoms of SRB fouling may precede their detection even when using the SRB- testers unless successful attempts have been made to disrupt these biofilms and cause the SRB to be forced out of the biomass and into the water. 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, SRB are viewed as being anaerobic and 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 derived from electron transport. SRB are defined as:

> "All bacteria that reduce forms of sulfate, elemental or organic sulfur to hydrogen sulfide, they usually are active under oxygen-free (i.e., reductive, anaerobic) conditions and use fatty acids (particularly acetate) as the main source of organic carbon"

While hydrogen sulfide (H_2S) is the main product of interest from the SRB the source of that gas appears to be either: (1) sulfate reduction; (2) the degradation of sulfur amino acids in proteins; or (3) from elemental sulfur.. Traditionally it has been considered that the H_2S originated solely

from sulfate reduction but, in practice, samples having significant protein content can also generate H_2S during reductive protein degradation in which ammonium is another major daughter product. A practical case could be made for replacing sulfate reduction as the primary feature in the bacterial generation of H_2S with "sulfide producing bacteria" since this would recognize both primary sources of sulfur (i.e. sulfate and sulfur amino acids in proteins) as having the potential to be reduced to H_2S.

In the practise of engineering it is common to use alloys incorporating iron into structures (for example, mild steel in gas, oil and water pipelines, and rebar into concrete). If these alloys become challenged by infesting biomass then there are distinct potentials for reductive conditions to become established at steel: biomass interfaces. This could readily lead to the generation of H_2S, electrolytic conditions and subsequent corrosion. It is generally believed that H_2S is a major trigger for electrolytic corrosion particularly at anodic sites where biomass tends to actively congregate. Additionally recent evidence using microbial fuel cells indicates that the bacterial communities within the biomass can generate direct current voltages in the range of 150 to 800mV at low levels of power. This phenomenon could be a major factor in the development of electrolytic corrosion. The common management practise used to control H_2S-influenced electrolytic corrosion is to apply cathodically impressed charges to the surfaces or coat with relatively impermeable or toxic durable coatings. When this is done then associable cost of these corrosion controls to microbiologically influenced corrosion (M.I.C.) can become very high. Generally, SRB are monitored as the prime indicator of M.I.C. risk but one major factor could relate to the practise of using sacrificial anodes. These anodes are placed to refocus the corrosive (biomass) activity away from the cathodically protected surfaces and materials. The risk here is that the bacterial communities involved in the corrosion processes now manipulate the electrical power associable with the sacrificial anode causing a refocusing of the location of that biomass.

To achieve selective detection of the H_2S generating (sulfide producing) bacteria including sulfate reducers and reductive proteolytic sulfide producers then the selective detection medium needs to incorporate sodium lactate, sulfate, and yeast extract along with the inorganic macronutrients. When prepared the sterile dried pellet weighs 241.2±1.3mg per tester.

Another major component in microbiologically influenced corrosion (M.I.C.) is acidulolytic corrosion caused primarily by acid producing bacteria (see APB in Chapter 3.5).

One major challenge in using the SRB- tester is dealing with samples that naturally contain H_2S. Once the H_2S concentration gets above 3ppm there can be a spontaneous reaction with black

sulfides forming in the tester. At >20ppm H_2S this black iron sulfide driven reaction becomes instant. Samples that are considered to have residual H_2S should be pre-treated to remove this gas from the sample to prevent early false positives in the SRB- tester. The easiest manner to drive off the gas is by vigorously aerating the sample for fifteen minutes prior to starting the SRB- testing. A small aquarium pump should provide adequate flows to remove the H_2S and a small cotton plug should be placed in the air line to reduce the possibility of contamination of the sample from the air being pumped.

3.3.2. Reaction patterns for the SRB- tester

There are two significant reaction patterns that can be interpreted as positive for the SRB- tester. These communities form the sulfide producing bacteria (SPB) in which there are two very distinct communities. Generally the reactions occur in sequence or only one of the reactions occurs. These are the only two recognized reactions although in the case of very active populations when the whole tester solution can turn black. This is called black all (BA) but is not recognized since it is a terminal reaction and has to have been preceded by one the reactions listed below:

- **Blackened Base (BB)**, this reaction is recognizable by the formation of a black deposit (Pantone black 6) first occurring in the basal cone of the tester. It may be first observed by looking up into the underside of the cone of the inner tester vial. Blackening frequently starts as a 2 to 3mm wide ring around the central peg and gradually spreads outwards. Eventually the blackening will spread to the bottom 3 to 5mm up the walls of the tester immediately above the cone. Declaration of a positive BB reaction should occur when there is either: (1) the formation of a completely jet black cone in the base of the tester; or (2) when black bands appear at the bottom of the walls just above the conical base. These are the sulfate reducing bacteria communities within the SPB.

- **Blackening at the top around the Ball (BT)** involves the formation of a slime ring may be viewed around the lower hemisphere of the ball with patches of black specking or zones intertwined in the slime growths. Initially the BART ball will show some grey zones (Pantone 5305 or 5315) often as an ellipsoid or banded. A positive should only be declared when granular jet black (Pantone black 6) granules appear in at least one sectors of the lower hemisphere of the ball. There may one or more biofilms (slimes) that form around this region of the ball before the black granulation starts. The slime itself is not a characteristic of this

reaction but the blackening is. The slime usually is either a white (Pantone 5315), grey (Pantone 5295), beige (Pantone 5015), or yellow (Pantone 584) color and tends to grow up onto the upper side of the ball. The blackening often begins as a specking which gradually expands to patches within the slime. Declaration of a positive BT should occur when the there is the first recognition of jet black granules or bands on the ball. These are the proteolytic sulfide producing bacterial communities within the SPB.

It is strongly recommended that no attempt be made to interpret any other forms of growth in the SRB-tester other than with direct respect to the BT and BB associated with the jet black (Pantone Black 6) reaction. Other (not significant) reactions range from cloud-like structures and thread-slimes in the colorless liquid medium or the formation of turbidity (clouding). Usually these structures form from the bottom up after which the growths will commonly expand to render the liquid medium turbid. These clouds are relatively stable structures and often have defined edges. These bacteria are functioning in a reductive regime since the tester does incorporate an oxygen blocker to reduce the diffusion of oxygen down around the ball. These bacterial growths are therefore anaerobic ferrmenters but these reactions are not recognized sine they do not relate specifically to any of the SPB. In summary the sulfide producing bacteria (SPB) are composed of the **sulfate reducing bacteria (SRB)** and the **proteolytic sulfide bacteria (PSP)** which together form the sulfide producers. This is summarised below as:

PSB = SRB (BB) + PSP (BT)

In which the reactions appropriate to the community are given in brackets within the equation.

3.3.3. Bacterial communities recognized by reaction signatures in the SRB- tester

There are only two reactions recognized for the SRB- tester and these are summarized below with a brief description of the dominant bacterial groups:

- **BB** may be considered to mean that there is a deep-seated anaerobic SRB infestation dominated by *Desulfovibrio* and these bacteria are commonly difficult to control due to the entrenched nature of the biomass;

- **BT** reactions indicate that there is dominant aerobic slime forming heterotrophic bacteria in the biomass which have active PSP present in the community. This reaction is more likely to

be associated with the generation of hydrogen sulfide from reductive proteolytic functions. In general control is more achievable since the PSP are more vulnerable in a larger biomass that may more easily be disrupted using a suitable chemical dispersant.

When observations are made daily in a very active SRB population then the BA can be the first positive reaction seen. However where the VBR I or II system is applied with the time lapse camera set to record every fifteen minutes then there is much better precision in the determination of whether the BB or the BT reaction precedes the BA. Without the use of the VBR then it could well happen that the initial BB or BT reaction that was not observed and a all-encompassing BA reaction then occurs and that is recorded with daily observations.

If the aerobic slime formers incorporate PSP into the growing biomass around the ball then these would be able to colonize under those anaerobic conditions generating a BT (not observed) reaction. Using the VBR I or II digital time lapse photographic methods it should be possible to differentiate the occurrence of BB and BT reactions before the BA reaction occurs. Where a BA reaction is first observed then the default would be to consider that reaction had started as a BB. From the practical point of view the reality is that the SRB- tester is really a test for sulfide producing bacteria. Thus positive detections can reflect H_2S being generated from sulfates, sulfur-containing amino acids, or elemental forms of sulfur. Sensitivity of the SRB- test to H_2S from elemental sulfur is constrained by the availability of other nutrients such as organics and phosphorus.

Unlike the IRB- tester that has a complex set of interacting reactions the SRB- tester is simple since it uses only one of two reactions (BB or BT) to differentiate the communities containing detectable SPB activity (as SRB or PSP). In general the BT reaction will be generated in samples where the ORP (oxidation reduction potential) values range between +50 and –20 millivolts (e.g. mildly oxidative) where there is either sufficient sulfate (>10ppm) or total organic carbon with a high proteinaceous content (>2ppm). Here there would be a significant aerobic (oxidative) biomass generated around the ball that could then generate reductive (fermentative) conditions deeper in the biofilms. It is at these sites that the SRB- tester could generate hydrogen sulfide utilizing either the sulfates or sulfur-proteins to generate a BT reaction. For the BB reactions which occur deeper in the cone at the base of the tester the sample is more likely to be reductive which would commonly involve ORP values between -20 and -150mv. Here conditions are more suitable for the reduction of sulfate using the fatty acids that are incorporated into the basal selective chemical nutrient pellet. BB reactions are more typical of deeply set covert SPB activities commonly located more deeply in

porous or fractured media. In general therefore the BB reaction signifies that the SRB infestation may be more difficult to treat effectively with the total elimination not achievable. Quality management of the SRB- tester is based upon one species and one natural sample. For the species, a culture of DSM1924, *Desulfovibrio desulfuricans*, is obtained using Sulfate Reducer (API) agar (based on the practise in *Recommended Practise for Biological analysis of Subsurface Injection Waters*, Volume 38, 2nd edition 1965. For the natural sample confirmatory testing the primary effluent (PE) from an aerated oxidative wastewater treatment plant may be used. Commonly the PE will generate the BT reaction in the SRB- tester with a 2% probability of a BB reaction occurring instead. SRB strain DSM1924 usually generates a BB reaction that will then move to a BA. This type of reaction (BB) also occurs when DSM1924 is mixed with ASTM27853 *Pseudomonas aeruginosa* (50: 50 with populations of each in the one to five million cfu/ml range). Primary effluent from an aerobic wastewater treatment plant has been found to reliably give a BT reaction which moves to a BA. Here the sample is used at full strength (15ml) and generates the BT moving to BA reaction in less than two days.

3.3.4 Time lapse determination of predictive active cell populations (pac/mL)

Both the culture of DSM1924 and the primary effluent were used as the sources for verification the SRB- testers and generation of the relationship between population and time lapse was performed using an earlier version of the VBR I system. To achieve these serial dilutions were now made using tenfold dilution with 1.5ml transfers made up with 13.5ml of SDW (sterile distilled water) into fresh sterile SRB- laboratory testers followed by thirty seconds of agitated mixing. Dilutions continued down to 10^{-7}. Incubation was at room temperature and the day that the first reaction occurred was used generate the time lapse relationship to the original population of SRB in the sample. Agar spread plate enumeration were included initially but tended to show a much lower sensitivity to the detection of SRB colonies than those obtained using the serial dilution method. Regression analysis of the cumulated data generated equation two gave the most suitable correlation:

$$y = -0.6378x + 6.977 \text{ (equation two)}$$

In equation two x is expressed as the time lapse in days and y is the predicted population expressed as pac/mL to the log base 10 and converted to population numbers in Table 3.3.1..

Table 3.3.1, Daily Observations of the SRB- BART tester showing variability in Predicted Populations (pac/mL)

	Time Lapse (days)			
	1	**2**	**3**	**4**
High population	8,180,000	1,800,000	434,000	99,900
Average population	**2,180,000**	**502,000**	**115,000**	**26,600**
Low population	283,000	134,000	30,800	7,100

	5	**6**	**7**	**8**
High population	23,000	5,290	1,220	280
Average population	**6,130**	**1,410**	**325**	**74**
Low population	1,630	376	96	19

3.4 Slime Forming Bacteria (SLYM- BART)

– Lime Green Cap

Parameter code: slime biotester

3.4.1. Introduction to the SLYM- Bart tester

Slime-forming bacteria (SLYM) are bacteria that are able to produce copious amounts of slime without necessarily having to accumulate any iron or other cations (scaling or encrustations). These slime-like growths are therefore not dominated by the yellows, reds and browns commonly seen when IRB- are dominant. Some of the IRB do also produce slime but it is often denser with more scale-like textures due to the accumulation of various forms of ferric iron, other cations and possible carbonates. SLYM bacteria can also function under different reduction-oxidation conditions generally producing the thickest slime formations under aerobic (oxidative) organic-rich conditions. These can sometimes develop in the SLYM- tester as slime rings growing around the floating ball but 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 bottom-up in the testers. One common check for these types of growth is to gently tilt the tester and see that the cloud- or gel-like growths retain their structure and position during the tilting of the tester. Definition of slime forming bacteria is summarized below:

> "Any bacteria that generate water-bonding polymers outside of the individual cells which take on the form of a coherent slime within which the cells can continue to remain active while being shielded by bound water"

Many bacteria can produce slime-like forms of growth when they generate biofilms and biomass which retain water. This bonded water is actually formed by a variety of extracellular polymeric substances (EPS) that are long thread like molecules. 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 slime volume is actually water and not bacterial cells. Some slime forming 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 body of slime. The role of the slime appears to be both protective and accumulative. Often this slime can be viewed as a common “skin” or “overcoat” to the cells and even nutrients are accumulated before being taken up by the cells! If environmental conditions are harsh (e.g., due to shortage of nutrients), then the slime layers tend to thicken. Not only does the slime act as a protectant to the resident bacteria but it also acts as a “bio-sponge” accumulating many chemicals that could form either a part of the nutrient base, or be potentially toxic to the cells if not bound into the EPS outside of the cells.

EPS may also be the site for chemical enzymatic activity (e.g., dextran sucrase or levan sucrase) degrading carbohydrates. Production of the EPS is actually within the bacterial cells and it is then extruded in the form an enveloping water-bonding slime. To achieve slime formation the SLYM- medium used consists mainly of proteose and peptone-tryptone form of proteins. This medium is an excellent broad spectrum culture medium that will support the growth of many bacteria with slime forming bacteria tending to dominate. Each SLYM- tester selective chemical nutrient pellet has a dried weight of 339.6±1.5mg per tester.

When the water sample or the porous semi-solid sample to be tested is added then there is added to the SLYM- tester there is dissolution and diffusion of the proteose and peptone-tryptone dried pellet vertically into the water column. This generates a concentration diffusion gradient that triggers the development of an oxidative-reductive interface which moves upwards at the same time as the nutrient diffusion front in the tester. This creates natural activity foci at the elevating interface and diffusion fronts. Such focal sites can trigger early visible growth reactions when the slime forming bacteria form as zones of growth often elevating with the medium diffusion front. These growths resemble slime plate-like discs that move upwards, divide, join together and then terminate with a cloud-like growth.

3.4.2 Reaction patterns for the SLYM- tester

Described below are the eight reactions recognized for the SLYM- tester. These are:

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 (Rare Reaction)

PB -Pale Blue Glow in U.V. Light

GY -Greenish-Yellow Glow in U.V. light

Of the above reactions, it is the CL (cloudy) reaction that is by far the most common. Often the CL will be preceded briefly by a CP which will be transient (lasting commonly less than 24 hours) and undergo constant changes from single disc-like plate to daughter plates which "rack" one above the other. Pantone colors are listed in section3.13.4. Descriptions of the major reactions including those involving UV fluorescent pigments are given below:

- **Dense Slime – DS** is a reaction that may not be obvious to the casual observer and could require a gentle rotation of the tester. When this is done slimy deposits will often swirl up if the test is DS. These deposits may swirl in the form of a twisting slime reaching up to 40 mm into the liquid column. Alternatively, globular gel-like masses form that settle fairly quickly afterwards. Once the swirl has settled down, the liquid commonly become clear again. In the latter case, care should be taken to confirm that the artefact is biological (ill-defined edge, mucoid, globular) rather than chemical (defined edge, crystalline, often white or translucent). Generally, DS growths are white, beige, or yellowish-orange in color.

- C**loudy Plates Layering – CP** is a reaction that can occur when there are populations of aerobic bacteria. Here the initial growth may form around the oxidative-reductive interface. It commonly starts above the yellowish-brown chemical nutrient pellet diffusion front. This growth usually takes the form of lateral or "puffy" clouds which is most commonly grey in color. Often the lateral clouds may consist of disc-like plates that are commonly relatively

thin (1 to 2 mm). It should be noted that if the observer tips the tester slightly then the clouds or plates will often move to maintain a constant position within the tester. These formations are most commonly observed 10 to 25 mm beneath the floating ball. Sometimes these plates will appear to divide laterally to form multiple plating. CP reactions usually terminate in a clouding of the medium (CL reaction).

- **Slime Ring - SR** is recognized as a 2 to 5 mm thick slime ring usually on the upper side of the ball beneath the water-headspace interface. 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.

- **Cloudy Growth – CL** is the commonest reaction and occurs when the solution becomes clouded (turbid.

- **Blackened Liquid – BL** is commonly a terminal reaction. It is recognized by the tester usually going black from the base up to the floating ball. The solution inside the tester is commonly as clear and colorless that has become surrounded by blacked walls. This reaction occurs under very reductive conditions with the formation of carbonized reduced organic compounds.

- **Thread-Like Strands – TH** occurs on limited occasions when the slime forming bacteria generate threads. These may be seen as complex slime threads that form between the base of the tester and the ball. These are temporary growths commonly lasting one to three days before dissipating to CL.

There are ranges of reaction pattern signatures (RPS) for the SLYM- tester but the dominant reaction most commonly seen is CL. Typical RPS include:

- DS – CL occurs when dense slime forming bacteria producing copious EPS, facultative anaerobes dominate;

- SR - CL tends to commonly occur when the sample is dominated by aerobic slime forming bacteria (such as M*icrococcus*)**;**

- CP - CL has been found to occur when the sample is dominated by motile facultatively

anaerobic bacteria (e.g., *Proteus*);

- CL - SR is a reaction that commonly occurs when the sample has a mixed bacterial flora (aerobic and facultative anaerobes) in which there are significant numbers of aerobic slime-formers;

- CL - BL involves a mixed community of slime formers dominated by Pseudomonads and Enteric bacteria that are able to function under very reductive conditions. There is a growing body of evidence that BL reactions can also be linked to organic carbon reduction to methane (4, 06-22 BNG) and elemental carbon (4, 05-17 COL);

- TH - CL indicates a dominance of slime forming aerobic bacteria which are able to generate slime threads (e.g., *Zoogloea*) during the early phases of growth,

- CL - PB occurs when *Pseudomonas aeruginosa* is a dominant member of the bacterial flora in the sample and generates a pale blue (PB) glowing reaction in the top quarter to one third of the tester and usually lasts between three to five day and then commonly fades away;

- CL - GY occurs when the *Pseudomonas fluorescens* species group is significantly present in the sample leading to a glowing greenish yellow (GY) reaction that extends downwards one third to a half into the tester. The glowing lasts commonly from three to ten days and, on some occasions, does not fade away.

3.4.3. Bacterial species recognized by reaction signatures in the SLYM- Tester

All of the reactions listed below in 3.3.3 utilize quarter strength brain heart infusion agar spread plates for the initial culture of the individual species followed by dispersion into sterile Ringers solution to obtain a cell population of 10^{7} to 10^{8} cfu/ml. 1.0ml of this suspension was then used to inoculate the laboratory version of the SLYM- tester with make up using 14ml sterile Ringers solution. Incubation for these reaction trials was at $22\pm2^{\circ}C$ with the reactions being generated sequentially within four days.

Bacteria from the American Type Culture Collection (ATCC) were used to develop quality management practices for the SLYM- BART tester. These are listed below with the ATCC number

bracketed before each species is followed by the reaction in bold:

8090 *Citrobacter freundii,* CL;
13048 *Enterobacter aerogenes,* CL-BL;
27853 *Pseudomonas aeruginosa,* CL-PB;
12228 *Staphylococcus epidermidis,* DS;
13315 *Proteus vulgaris,* CP-CL;
13883 *Klebsiella pneumoniae,* SR-CL;
25922 *Escherichia coli,* CL-BL;

3.4.4 Time lapse determination of predictive active SLYM- cell populations

To determine the relationship between time lapse at 22±2 ^{o}C and the cells populations inoculated into the tester, pure cultures of the bacterial species (see 3.3.3 above) were inoculated into SLYM- testers using tenfold dilution down to 10^{-7} using sterile Ringers solution using 1.5mL of the diluted inoculum in 13.5mL of solution. Concurrently comparable dilutions were performed using the agar spread plate technique employing 1.0mL of the diluant. Analysis was applied to the generated data to determine the "best fit" between the time lapse and the recorded population. Natural samples known to contain slime forming bacteria were also subjected to regression analysis. Here the regression analysis of the data found that equation three gave the most acceptable correlation:

$$y = -0.708x + 6.947 \text{ (equation three)}$$

In equation three x is expressed as the time lapse in days and y is the predicted population for slime forming bacteria expressed as log base 10. Table 3.3.4.1 gives the relationship of time lapse (in days) to the population. Here the calculation is based upon the range of populations that could have been generated during the time period of 24hours that the time lapse could have been observed. For example a three day (72 hours) observation would have a maximum variation from 49 to 95 hours. The calculation of the population could therefore vary from the average (72hours) from a low (49hours) to a high (95 hours). The range in Table 3.3.4.1 illustrates this variability.

Table 3.4.4.1, Daily Observations of the SLYM- tester showing variability in Predicted Populations (pac/mL)

	Time Lapse (days)			
	1	**2**	**3**	**4**
High population	7,510,000,000	1,470,000	288,000	56,500
Average population	**1,730,000,000**	**339,000**	**66,500**	**13,000**
Low population	399,000	78,300	1,630	3,000
	5	**6**	**7**	**8**
High population	11,000	2,160	424	83
Average population	**2,550**	**500**	**97**	**19**
Low population	588	115	22	4

3.5 Heterotrophically Active Bacteria (HAB- Bart Testers) – Blue Cap

Parameter code: bacterial biotester

3.5.1. Introduction

Some bacteria are able to degrade organics as their source of energy and carbon. These are known as heterotrophically active bacteria ("organic busters" or "general HAB bacteria"). By far, the majority of these heterotrophs function most efficiently under aerobic (oxidative respiratory) conditions. Many of these bacteria will also function albeit less efficiently under anaerobic (reductive fermentative) conditions. Since these bacteria play a major role of biodegradation of organics-of-concern, their presence in oxygen-rich waters is often critical to the efficiency of many oxidative (aerated) engineered operations.

HAB- testers were developed to detect the "organic busters" under both oxidative and reductive conditions. In this test the unique feature is the addition of methylene blue which colors the liquid samples blue under oxidative conditions and clear under reductive conditions. It functions as an ORP (oxidation-reduction potential) indicator well over a broad pH range from 1.5 to 14 but under very acid conditions it will lose the blue color. This means that methylene blue acts as an indicator of bacterial activity being blue under respiratory (oxidative) and clear under fermentative (reductive) conditions. Generally the "organic busters" work more efficiently under oxidative conditions (generating carbon dioxide). Under reductive fermentative conditions the daughter products are fatty acids. While there remains free oxygen in the tester the methylene blue dye in the water will remain blue. As soon as all of the oxygen has been consumed (residual oxygen falls below 0.04ppm by bacterial respiratory) then the methylene blue shifts from its observable blue form to a colorless form. In other words, in the HAB- testers, when the liquid medium turns from blue (Pantone Proc Blue CS) to a colorless (non-blue) form, then the heterotrophic aerobic bacteria have been sufficiently aggressive to have "respired off" the oxygen. It should be noted that the water sample may interact with the blue shifting the color towards blue-green (Pantone 3145 CS) in the event of higher salt concentrations. If the salt concentration exceeds 1% or the total petroleum hydrocarbons (TPH) exceed 100mg/L then it would be necessary to pre-dissolve the methylene blue dried in the cap using 1.0ml of sterile

distilled water (SDW). To do this add 1.0mL of SDW to the inside of the cap and leave for one minute to allow the dye to dissolve. Note that the one mL SDW will just fill the inside portion of the cap where the methylene blue has been dispensed as a dried crystalline deposit.

There is a oxidative to reductive color shift (blue to clear) which works effectively over a pH range from 1.5 to 14.0. Very acidic water will also cause the water to shift from blue to clear since methylene blue acts as a pH indicator when the pH of the sample is <1.5 pH units. This may be mistaken for a positive reaction when it is really the methylene blue acting as a pH indicator. Samples with a natural pH of less than 2.0 should have the pH adjusted upwards to greater than 3.5±0.2 using sterile N KOH at the start of the test before the methylene blue is mixed. Another interference factor that could cause absorption of the methylene blue is high concentrations (>100ppm) of petroleum hydrocarbons and this can also be corrected by predissolving the methylene blue in the cap. Definition of general HAB bacteria is summarized below:

> "Any heterotrophically active bacteria includes those that can generate an active biomass through degradation of the organic materials in the sample by respiration (oxidatively) or fermentation (reductively). This activity is monitored using the shifting in the oxidation-reduction potential which creates an oxygen demand that is observed using methylene blue as the indicator"

When active bacteria cause reductive conditions to develop due to the consumption of the dissolved oxygen then the blue color will become bleached out. This usually occurs from the bottom (up, UP) for aerobic bacteria; or from the top (down, DO) in the tester if anaerobic bacteria dominate activities. Note that the methylene blue indicator dye is added in the tester's cap and starting the test is achieved by inverting the charged HAB- tester for 30 seconds, rotating three times then to allow the chemical dried methylene blue in the cap time to dissolve into the sample under test. When the HAB- tester is returned to its normal state (cap side up) and the wrist action rotation (x3) of the tester now causes the ball to roll through the water sample allowing the methylene blue to become more evenly mixed into the sample to form an blue solution. At the same time the sample below the floating ball becomes saturated with headspace oxygen from above the ball.

Methylene blue is a basic dye that can readily bind to the negatively charged microbial cells. Traditionally, therefore, this dye has been used to stain microbial cells. When methylene blue is added to a medium at the very start of the test then there is an active conversion of energy due to microbial respiration due to the demand for oxygen. 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 HAB- 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-tester the objective is for the user to be able to determine the active 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 bacterial activity in the sample.

To achieve the activity of the heterotrophic bacteria the selective culture medium used is composed of a very rich medium of proteose and peptone-tryptone along with other important macronutrients. This medium is an excellent broad spectrum culture medium that will support the growth of many bacteria with slime forming bacteria tending to dominate. Each HAB-tester contains a selective chemical nutrient pellet has a dried weight of 349.1±1.2mg per tester when combined with the methylene blue dried into the cap. When the sample is added then there is dissolution and diffusion of the proteose peptone-tryptone dried pellet as it dissolves vertically into the blue water tester column.

When the HAB- tester was originally developed (1988 to 1993) it was considered that the tester was only effective for detecting aerobic bacterial activity and the name “heterotrophic aerobic bacteria” was adopted. From 1998 to 2005 it was found that the test was also very effective for the generalised detection of bacterial activity and the name was changed to “heterotrophically active bacteria” to recognize the anaerobic activity that can also be associated with this tester. From 1996 to 2012 the potential role of the HAB- for monitoring the oxygen demand in the biochemical oxygen demand (BOD) test which takes five days was evaluated There is now new protocol / strategy (rBOD and pBOD) that are described in subsequent chapters in this Standard Methods.

Unlike the other BART testers that operate principally at 22±2^{o}C the HAB- tester can also be operated at a variety of incubation temperatures using the VBR I and II systems. While the VBR I system has to be operated at room temperature or in an incubator, VBR II allows the temperatures to be pre-set to 4±2^{o}C*, 12±1^{o}C*, 20±1^{o}C, 22±1^{o}C, 28±1^{o}C 37±1^{o}C, 45±1^{o}C, and 54±1^{o}C and 62±1^{o}C. For the lower temperatures (*) the VBR I system would need to installed in a temperature controlled incubator or room. For temperatures above 23^{o}C then the VBR II system could be employed using regular laboratory temperatures.

Conducting an HAB-test involves a set of methodologies that would ensure that the methylene blue (dried into the cap) mixes easily with the prepared liquid sample. For water samples that have been verified to have a pH of greater than 6.5 then the protocol is straight forward:

(1) Unscrew the cap on the tester and lay cap down on clean dry surface;

(2) Add 15ml liquid sample to the fill line and the ball will float up;

(3) Screw the cap back down on the tester;

(4) Turn the tester upside down and leave for 30 seconds to allow methylene blue to dissolve;

(5) Turn the tester right side up and by slow wrist action rotate the tester three times to mix the methylene blue into the solution; and

(6) Now begin test.

If a semi-solid or solid sample is to be tested then the protocol changes since particulates from the sample could cause a jamming of the ball lower down in the tester:

(A) Unscrew the cap and place down open-side up;

(B) Roll out the ball into the cap;

(C) Add the semi-solid or solid sample to the tester;

(D) Add sterile distilled water to make up the total volume added to 15ml;

(E) Roll the ball back into the tester so that the ball now floats at the fill line;

(F) Add 1ml of sterile distilled water to within the inner flanges of the cap (which has a capacity of 1.15mL) and leave for 30 seconds;

(G) Pour contents of cap over the floating ball; and

(H) Screw down the cap onto the tester, do not conduct rotational agitation and start test.

Note that step F is also required of samples that have a salt content of greater than 1% (v/v) or TPH of greater than 100mg/L.

3.5.2. Reaction patterns for the HAB- Tester

There are only two major recognized reactions (UP and DO) and both of these relate to the form with which the bleaching occurs. These are defined as:

- **UP reaction** where the bleaching moves upwards from base of the tester. Here the blue solution in the tester 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 (Pantone 7401 to 7404). Rarely does the bleaching extend up beyond the equator of the ball so that a blue ring will commonly remain around the ball with a width of 1 to 5 mm. An UP reaction is typical of the strictly aerobic bacteria but there also may be facultative anaerobic bacteria also present.

UP reactions routinely occur from the top of the cone in the base upwards. Commonly (>80% of the time) these UP reactions can be seen by the formation of a sharp lateral line with blue (oxidative) conditions above and clear or light yellow (reductive) conditions below. The rate at which this front moves up the tester to 5mm beneath the floating Bart ball varies with the amount of bacterial activity. Normally the rates range from 10mm/hr down to 2mm/hr. Where there is very high activity (such as in primary influent to a wastewater treatment plant) then there will still be an UP reaction but the bottom 20 to 35mm in the HAB- tester may fade from blue to clear concurrently. The CBR software is able to detect this using the two calibration circles set at 20 and 25mm. Precision can be gauged undertaking replication of the sample in multiple testers and commonly the variance is less than 5%. On a limited number of occasions the UP reaction may start but then stall and even reverse turning the tester solution in the base back to blue. This can

happen when there is some inhibitory material in the sample that reacts negatively with the respiring bacteria causing stasis (shut down) in the bacterial community. Once this happens then oxygen from the headspace may now penetrate back to the base of the tester it blue again. This has not been significant in treated wastewater but has occurred in industrial wastewaters where there could be significant toxic materials in the influent.

- **DO reactions** occur when the bleaching moves down. Here the blue solution bleaches from the top of the tester down. Commonly bleaching is more clouded and initially indefinite when compared to a typical UP reaction. 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. DO reactions are dominated by facultatively anaerobic heterotrophs but some strictly anaerobic bacteria may also be present. This reaction is generally slow to form and a positive should not be declared until the blue color has disappeared 30mm up from the base of the tester.

DO reactions are less common during HAB- testing. When these types of reactions occur then the primary factor that declares the DO reaction is the development of a fading blue zone below the ball. Generally these types of reductive activities between the bottom of the Bart ball (42mm) and the midpoint (25mm) up from the base of the tester. While the UP reaction is generally stable and then rises steadily the DO reaction rather resembles clouds that appear and then disappear. Such an event would be linked to the variable oxygen demand being created by the initially unstable fermenting bacteria in the sample. In the CBR software it is recognised that there would commonly be some instability in the reductive activities that would render the generation of time lapses that would have little precision. Consequently the declaration of a DO reaction is established when the calibration circle at 25mm declares at least a 40% loss in the blue color within the target pixels. Time lapse declaration using the CBR then occurs after the initial (and potentially unstable generations of reductive zones) and only when the DO reaction has stabilised. DO reactions are very rare (<2%) in primary influent samples tested from municipal wastewater treatment plants. In practise DO reactions are more likely to occur in samples taken from bioremediation sites, deep groundwater sources and from industrial land fill operations.

In testing samples using the HAB- tester there is a risk of interaction between the chemistry in the sample and the diffusing chemistry associated with the dissolving chemical nutrient pellet in the base cone of the tester. Commonly this causes the blue color to shift to a bluish green hue (Pantone 3145). These types of color reaction have been found not to affect the performance of the HAB- tester

3.5.3. Bacterial consorms recognized by reaction at 22±2^{o}C in the HAB- tester

Two ATCC bacterial strains 27853, *Pseudomonas aeruginosa;* and 25922, *Escherichia coli* were used to define the reactions UP and DO respectively. Spread plate populations were obtained from these cultures and also from natural samples using 0.1mL dilutions in sterile distilled water down the 10^{-8} with streaking out on quarter strength brain heart infusion agar, incubation at 22±2^{o}C for seven days using thick 25±2ml agar inside thick sterile polyethylene bags to reduce moisture loss. Colony counts of between thirty and three hundred were used to calculate the population of HAB. Thirty natural water and soil samples were utilized along with the pure cultures to calculate the relationship between the time lapse using the HAB- BART tester and the predicted population at 22±2^{o}C. One third of the natural samples came from reductive environments (giving DO reactions) while the remainder came from oxidative environments (giving UP reactions).

3.5.3.1. Time lapse determination of predicted active cell populations using HAB- tester

HAB- testers have proven to be very effective tools working with the VBR and CBR software at temperatures ranging from 4±2^{o}C*, 12±1^{o}C*, 20±1^{o}C*, 22±1^{o}C, 28±1^{o}C 37±1^{o}C, 45±1^{o}C, and 54±1^{o}C and 62±1^{o}C using versions of the VBR II system. Where lower temperatures are to be achieved than the VBR II unit is placed in a temperature controlled cooled incubator (see *). All of these temperatures have supported activities generated by the HAB- tester. Examples include: (1) 4±2^{o}C has been used to monitor bacterial HAB activity within the ice flows affected by treated effluent discharges into the arctic ocean; (2) 12±1^{o}C has been used on occasions to monitor HAB

activities associated with bioremediation sites where TPH and BETEX are of concern; (3) 20±1 ^{o}C has been used to examine HAB activities in a variety of environments but higher temperatures have been found to generate faster results; (4) 22±1 ^{o}C the common laboratory/office temperature has been used for convenience since no incubator is required and precision has proven acceptable; (5) 28±1 ^{o}C has proven to give shorter time lapses than at room temperature but there has been some losses in precision when operating at this temperature; (6) 37±1 ^{o}C is a very popular temperature for incubating suspected pathogens in samples from warm blooded animals. While generally the pathogens in the sample do exhibit some precision any environmental bacteria will generate instability increasing variance; (7) 45±1 ^{o}C is a temperature which excludes the activity of many environmental bacteria and also some pathogens with the result that the incubation temperature will detect limited numbers of specialised pathogens (e.g. fecal E coli), (8) 54±1 ^{o}C and 62±1^{o}C are higher temperatures that usually will restrict the types of bacteria growing to only those adapted to those temperatures. Examples of this would be in heat exchanger systems, solution extraction processes involving heat, environmental sites being impacted by hot effluent discharges.

A major result of the recognition of the very considerable adaptability to a wide range of temperatures has been the election of two temperatures (22±1 ^{o}C, and 28±1 ^{o}C) for the development of links between the time lapse and the predicted populations as expressed in pac/mL. There are three conditions recognised at this time. These are 22±1 ^{o}C for environmental bacteria and 28±1 ^{o}C for wastewater bacteria and also bacteria in environmental conditions where some heating effects are observed (e.g. composting, exothermic degradation, and low levels of heat inputs from industrial processes). There are three formulae generated based on room temperature (3.5.3.2), wastewater treatment (3.5.3.3) and warmer environmental conditions (3.5.3.4).

3.5.3.2. Time lapse determination of predicted active cell populations using HAB- tester incubated at 22±2^{o} C

Regression analysis of natural water and soil samples incubated at 22±2 ^{o}C data found that equation four gave the most suitable correlation between the time lapse (x, days) and the predicted active cell population (y):

$$y = -0.9737x + 7.706 \text{ (equation four)}$$

In equation four x is expressed as the time lapse in days and y is the predicted population expressed as the log base 10 (Table 3.5. 1.)

Table 3.5.1 Daily Observations of the HAB- tester showing variability in Predicted Populations (pac/mL) using 22±2 ^{o}C

	Time Lapse (days)			
	1	**2**	**3**	**4**
High population	40,600,000	4,310,000	458,000	48,700
Average population	**5,390,000**	**573,000**	**60,900**	**6,470**
Low population	717,000	76,200	8,100	860
	5	**6**	**7**	**8**
High population	11,000	2,160	424	83
Average population	**2,550**	**500**	**97**	**19**
Low population	588	115	22	4

3.5.3. Bacterial consorms recognized by reaction at 28±1 ^{o}C in the HAB- Tester (municipal wastewater)

Three pure cultures (*Escherichia coli*, ATCC #25922; *Klebsiella pneumoniae*, ATCC #13315; and *Pseudomonas aeruginosa*, ATCC #27853) were used in three trials with six replications for the brain heart infusion agar spread plates with three replicates for the WW-HAB- tester system at each of ten dilutions of the cultures being examined. All three species gave good correlations (R>0.9) but displayed different slopes and intercepts. Therefore for the calculation of the equation for converting time lapse to population (as predicted active cells per ml) all of the data was pooled to generate a mixed pool of the data from the three pure cultures and these were used to generate the population for municipal sanitary wastewater treatment samples. In comparative spread plate enumeration trials it was found that the pac/ml generated from the VBR 18 version at 28±1^{o}C and matched with the colony forming units that were generated by the traditional

spread plate technique. VBR 28 is normally used for the determination of heterotrophic populations in sanitary wastewaters. The VBR 28 can also be used to determine populations in environmental samples using E-HAB testers at 28±1^{o}C but the growth rate was slower and is discussed later in section 3.4.5. The VBR I or II can be used for the determination of populations incubated at 22±2^{o} C and are discussed in section 3.4.3. While the time lapses are shorter at this temperature there is generally less precision than at room temperature.

3.5.3.1 Time lapse determination of predictive active cell populations, WW - HAB- Testers (28±1^{o}C) .

For the prediction of active cell populations incubated at 28±1^{o}C there are two scenarios presented. The first scenario is specifically related to the general HAB bacteria that are found in domestic sanitary wastewaters (WW) and the predictive system employed is the WW-HAB- tester. The nature of these WW- bacteria is that they are well adapted be very active and generate large populations. The second scenario (environmental, E) is for samples that might not be quite as active as the WW but still generate greater levels of activity and populations sizes over time compared to 22±2^{o}C. There are two equations for the operation of the WW-HAB- testers incubated at 28±1^{o}C. Equation five covers time lapses of up to 24 hours and is set in seconds (86,400). Should the time lapse exceed 86,400 seconds then the calculation of population (y, pac/ml) utilizes equation six. Both equations give the correlation between the time lapses (x) registered in seconds and y as the predicted population (pac/ml) expressed as the log base 10.

$$y = 10^{((-0.00005x) + 11.1)} \quad \text{(equation five)}$$

$$y = 10^{((-0.00006x) + 11.26)} \quad \text{(equation six)}$$

In these equations, equation six is used where the time lapse (x) in seconds was found to be <86,401 while equation seven is employed only when the time lapse (x) was >86,400. Table 3.4.3.2 shows the relationship between time lapse (x, seconds) and the calculated predicted populations (pac/ml) using both equations. Table 3.4.4.1 shows the predicted populations that would be recorded at 4, 8, 16, 24, 32, 40, 48, 64 hours including the high low populations that could occur should observations only be made at those times. For the WW-HAB- tester with the usual time sequence for the VBR30 being set at every fifteen minutes (0.25hour) and so the

precision would then be much improved. Note that table.3.5.2 shows hours for the time lapses assume readings are only taken at the hourly intervals shown above and so the precision is limited by the frequency of the observations. If the VBR 28 is used with digital images of potential reactions every fifteen minutes then this would affect the upper table using equation five and the lower table (*) using equation six. Essentially high and low populations would now be calculated from time lapse using the 15 minute intervals (0.25hours) between images and so deducting 899 seconds for the high and adding 899seconds for the low population would give more precision to the range. Shortening the intervals to less than 900 seconds would increase the precision.

3.5.3.4. Time lapse determination of predictive active cell populations as pac/mL, environmental HAB- BART (28±1 ^{o}C)

For the prediction of populations of general HAB bacteria for samples from environmental origins using the VBR 28 with an incubation temperature of 28±1 ^{o}C then a different formula is employed (equation seven). This formula seven recognises the fact that HAB bacteria of environmental origins do not have such metabolically adapted populations as found in wastewaters. As a result the relationship between time lapse and predicted population is very different and the VBR 28 recognized this using the E-HAB- tester software. Essentially the E-HAB software functions for the full eight days of incubation (rather than the two days used for the WW-HAB software). There is a single equation (seven) that allows the prediction of the active cell population:

$$Y = (9{,}000{,}000.\ X)^{-4.82} \qquad \text{equation seven}$$

Where time lapse (x) is expressed in days and fractions thereof and y is the predicted active cell population per mL (pac/mL). These relationships are shown in Table 3.5.3 with the assumption that readings are taken daily for eight days.

Table 3.5.2 Selected Hourly Observations of the WW-HAB- tester showing variability in Predicted Populations (pac/mL) using 28±1 ^{o}C with observational readings in hours.

	Time Lapse (hours)			
	4	**8**	**16**	**24**
High population	83,100,000,000	15,830,000	3,010,000	109,000
Average population	**23,900,000**	**4,570,000**	**165,000**	**6,020**
Low population	6,910,000	251,000	9,120	37
	32	**40**	**48**	**64**
High population	724,000	16,000	253	4
Average population	**22,000**	**416**	**7**	**1**
Low population	1,000	12	1	1

Table 3.5.3. Selected Hourly Observations of the E-HAB- tester showing variability in Predicted Populations (pac/mL) using 28±1 ^{o}C with observational readings in hours.

	Time Lapse (hours)			
	1	**2**	**3**	**4**
High population	100,000,000	5,700,000	250,000	38,000
Average population	**9,000,000**	**320,000**	**45,000**	**11,020**
Low population	408,000	53,000	13,000	4,000
	5	**6**	**7**	**8**
High population	10,000	3,500	1,500	710
Average population	**4,000**	**1,500**	**750**	**400**
Low population	2,000	800	400	240

Note: precision can be achieved using the VBR 28 system set to record once an hour and so deliver 192 images and improved precision in the determination of the E-HAB bacterial population. This precision can be increased by shortening the interval.

3.6 Acid Producing Bacteria (APB- BART)

- Purple Cap

Parameter code: acidogenic biotester

3.6.1 Introduction

There are two major groups of acid producing bacteria that are most commonly associated with various corrosive events. One group generates inorganic acids (particularly sulfuric acid) from the oxidation of sulfides or elemental sulfur. These are known as the aerobic sulfur oxidizing bacteria and would not be detected by the APB- tester. It is the second group which are (organic) acid producing bacteria (APB-) that are detected by this tester. These APB cause the pH to drop significantly from neutral to acidic conditions giving terminal pH levels from 4.0 to 5.5. Commonly corrosion is linked to the generation of hydrogen sulfide which smells like rotten eggs and is thought to initiate the electrolytic corrosion of iron in steels and other alloys. Commonly black sulfides accompany the corrosion event which makes linking corrosion to H_2S and the sulfate reducing bacteria a logical extension. Corrosion can cause pitting leading to perforation and also erosive forms of corrosion that cause a generalised thinning of steel plates and increases in the porosity of the steel as pipes carrying water. Links between pitting and perforative forms of corrosion and the sulfate reducing bacteria and the SRB- tester was designed to detect the common sources of the H_2S. However erosive forms of corrosion can also be very significant factors affecting the operating life span of steel pipes. Erosive corrosion involves a stead lateral thinning (or dishing) of the steel until there is either perforation (causing catastrophic discharge) or increases in the porosity of the steel leading to "weeping" and then generalised failure with no specific perforation involved.

From past practises it has been found that erosive form of corrosion may not involve sulfate reducing bacteria (rather the sulfide producing bacteria (see section 3.2.2) but do involve the acid producing bacteria that under anaerobic (reductive) conditions fermenting organics with fatty acids as a major set of daughter products. These fatty acids locally reduce the pH into the mildly acid range (4.0 to 5.5) which then structurally compromises the strength of the impacted steel. In this context the acid producing bacteria (APB) are restricted to the fermentative reductive anaerobic bacteria who generate organic fatty acids as daughter products. Corrosion in

fire sprinkler systems is now commonly recognised to be dominated by the APB communities. In the oil and gas sector there is a growing realisation that the APB may be very significant "players" in the cause of corrosion in that industry too!

APB activities are formed by a variety of heterotrophic bacteria that share the common fermentative ability to produce organic (fatty) acid products when growing under reductive conditions utilizing organics. These mildly acidic products are sufficiently corrosive impact the integrity of many metallic alloys (e.g. particularly those with a high aluminum component). Because these acid-producing activities occur in the absence of oxygen, it has been found that the APB are very likely to be significant partners in corrosion with sulfate reducing bacteria (SRB) particularly in the oil and gas industry. As a result the management and control of corrosion frequently involves assessing the aggressivity of both the APB as well as the well-recognized SRB. As a result of industrial practices over the last century it has always been considered that microbiologically influenced corrosion (MIC) events were dominated by the sulfate reducing bacteria (SRB) because of their ability to trigger electrolytic corrosion in the metals through H_2S. SRB corrosion events occur primarily under highly reductive conditions in the presence of adequate sulfates and organics. In general the SRB generates hydrogen sulfide (H_2S) as a metabolic product and it is this gaseous product that then triggers the electrolytic corrosive processes. Some MIC occurs under mildly acidulolytic conditions. Acidulolytic corrosion appears to be caused by bacteria (e.g. APB) that are able to generate mildly acidic products generally under highly organic and reductive conditions with organics present. Note that the APB- tester was not designed to detect the acetic acid bacteria group and these bacteria tend to dominate under more oxidative conditions in the presence ethyl alcohol and sugars with the acetic acid being a product of that metabolism. Definition of the acid producing bacteria is defined here as:

> "Acid producing bacteria are defined as being capable of fermentation of organics under reductive conditions with daughter products including fatty acids. It is these short chain fatty acids that lower the pH within the immediate environment and trigger acid-related erosive forms of corrosion."

In the environment these daughter product fatty acids are reductively degraded to H_2S in the presence of sulfates and ORP in the range of 0 to -150mv and under more reductive conditions to methane (CH_4). Today it is recognized that the APB are significant contributors to corrosive MIC processes. In these cases then the compromises are likely to be through the gradual dissolution of the metal under the mildly acidic conditions that are created (e.g. shallow lateral dishing, erosive corrosion, of steel walls and gradual losses in strength commonly along with increases in relative porosity). In general the APB has been found to be active under reductive conditions within biofilms, slimes, patinas, encrustations, nodules and tubercles. Their activity can sometimes be noted as a lateral erosion of the metal surface that can be most clearly seen when the metal surfaces are examined using reflective light. Much of the APB is usually located directly at the metal – biomass interface under the growing biomass. If present, the surface of the metal will appear to have an irregular pattern of shallow depressions (dishing). This would mean that the most effective examination of a sample for the presence of APB would be achieved by sampling the slime / concretion / patina / encrustation / nodule / tubercle immediately at the interface between the biomass growth and the metal surface. This is different to the electrolytic corrosion caused by SRB which tends to cause deeper electrolytic pitting of the metals and deeper profiles to the cavities. In simple terms the APB generally cause broad impacts over much of the metal surface while the SRB cause focused forms of pitting and perforation of the metal.

To generate acid conditions through the production of the shorter chained fatty acids, the selective culture medium pellet in the APB- tester is rich in proteins and glucose and the individual pellet also contains tryptone, peptone, and glycerol along with the basic macro-nutrients and weighs 484±30mg/tester. This weight includes the pH indicator, bromocresol purple, which is deposited as a dried crystalline deposit in the inner cap of the APB- tester. One cautionary note is that the APB- tester functions through the pH dropping from purple (Pantone 513) into the acidic range as a result of the fermentative activities in the sample generating fatty acids. It is therefore important that the pH of the sample be determined before beginning a test to ensure that the sample being tested has a pH of greater than 6.5. Samples with a pH of less than 4.5 will go yellow immediately and samples over the range of 4.6 to 6.5 are likely to give a premature short time lapse**.**

Note: It is strongly recommended that samples with a pH of less than 6.5 be adjusted using

sterile one normal NaOH to within the range of 6.6 to 7.2.

Conducting an APB- BART test involves a set of methodologies that would ensure that the bromocresol purple (dried into the cap) mixes easily with the prepared liquid sample. For water samples that have been verified to have a pH of greater than 6.5 then the protocol is straight forward:

(1) Unscrew the cap on the tester and lay cap down on clean dry surface;
(2) Add 15ml liquid sample to the fill line and the ball will float up;
(3) Screw the cap back down on the tester;
(4) Turn the tester upside down and leave for 30 seconds to allow bromocresol to dissolve;
(5) Turn the tester right side up and by slow wrist action rotate the tester three times to mix the bromocresol purple into the solution; and
(6) Begin test.

If a semi-solid or solid sample is to be tested then the protocol changes since particulates from the sample could cause a jamming of the ball:

(A) Unscrew the cap and place down open-side up;
(B) Roll out the ball into the cap;
(C) Add the semi-solid or solid sample to the tester;
(D) Add sterile distilled water to make up the total volume added to 15ml;
(E) Roll the ball back into the tester so that the ball now floats at the fill line;
(F) Add 1ml of sterile distilled water to within the flanges of the cap which has a capacity of 1.15ml and leave for 30 seconds;
(G) Pour contents of cap over the floating ball; and
(H) Screw down the cap onto the tester, do not conduct rotational agitation and start test.

If the pH of the sample under test is even mildly acidic (e.g. <6.5) then it is recommended that the sample be brought up into the range that is in the 6.5 (minimal) to 7.4 (optimal) using sterile N NaOH solution.

3.6.2. Reaction patterns for the APB- tester

There is a single reaction pattern recognized for the APB- tester that causes by the pH indicator, bromocresol purple to shift from purple to yellow. This is referred to as the dirty yellow (DY) since activity associated with fermentation generates a significant biomass with numerous daughter products. The reaction may begin around the BART ball, in the sample, or from the basal pellet moving upwards. This DY reaction can be clearly recognized by the generation of a dirty yellow (Pantone 127 to 129) patch that then expands to greater than 80% of the liquid in the tester. It may be noted that before the recognition of the DY reaction the purple color (Pantone 513) in the tester may change to a lighter shade of purple (e.g. Pantone 514). This should not be considered as a positive test, it is only when DY expands to greater than 15% of the tester's solution volume that the reaction should be considered positive. This initial reaction may occur anywhere in the testers solution. On rare occasions it even appears first above the Bart ball but most of the time it is in the bottom half volume of the culturing solution.

For many samples being tested the APB within the sample may react in a way to secondarily buffer out the pH back to neutral. This buffering effect would mean that the tester would return from DY to a shade of purple. This is called the DYB (dirty yellow color buffered back to neutral The VBR I and II system are excellent for catching the time when this reversal occurs. It is more common to see the buffering extending from the bottom upwards, top downwards or as a series of expanding patches when it happens. For the APB- tester it is therefore very important to monitor the test sufficiently frequently (e.g. every six hours, use the VBR I or II system with time lapse frequency set at every 15 minutes). Buffering is not recognized as a recordable reaction for APB. However under some conditions the bacteria within the incubating sample will buffer the pH back into the neutral range and the APB- test will again shift to a shade of purple (buffered reversal).

3.6.3. Bacterial reaction signatures in the APB- tester

Acid producing bacteria technically are subdivided into two groups based upon whether they generate inorganic or organic acids. For the former group the dominant acidic product is sulfuric acid (from sulfur oxidation) while for the latter group the dominant acid products are fatty acids. APB for the purposes of this application is limited to the latter group which generates fatty acids from the reductive degradation (fermentation) of more complex organic compounds. These bacteria are fundamentally facultatively or strictly anaerobes which have the ability to degrade a multiplicity of organics to shorter chained organic acids as principal end products that then lower the environmental pH. There is only one reaction pattern (DY, dirty yellow) recognized and the buffering function that frequently occurs to shift the pH back to a neutral range is recognized as DYB. However it should be noted that a purple APB- tester does not mean after four or five days that it is negative but it can also mean that the acidic reaction has become buffered out. Generally the time when the DY can be observed last one to three days before any buffering occurs. It is therefore important to minimally observe the test daily for reactions or use the VBR I or II systems.

For the ubiquitous fermenting bacteria under reductive conditions normally will generate some level of organic acidic daughter products. This would mean that a broad spectrum of facultative and strictly anaerobic bacteria will be acid producers. Standard trials use species within the enteric bacteria group preceded by ATCC numbers: 8090, *Citrobacter freundii*; 23355, *Enterobacter cloacae*; and 13315, *Proteus vulgaris*.

3.6.4. Time lapse determination of predictive active cell populations APB - tester

Due to the wide abundance of APB in the reductive (fermentative) environments the correlation between time lapse and population was prepared using natural samples. Populations were assessed using serial dilutions of the samples down to 10^{-9} using sterile distilled water (SDW) as the diluant and 1.5ml as the transferred volume between diluents. Each completed diluant should

then be transferred to APB- testers which for incubation at room temperature (22±2 ^{o}C) for eight days. These should now be observed generation of a DY reaction and the time lapse noted. For greater dilutions there activity might be delayed and ten days of incubation may be necessary to assure that no APB were detected. Using natural samples which were mostly semi-solid porous relatively fragile structures (e.g. patinas, ochres, rusticles, encrustations, tubercles, and pitted material) these were dispersed using commonly 0.5g of material into the diluant Ringers and then subjected to dilution and incubation. Confirmation was obtained by subculture 1.5ml of positive DY into a fresh tester and incubating to confirm the DY reaction. Colony forming units were recorded by spread plate analysis using 0.1ml of each diluant on R2A agar with incubation for ten days at room temperature. Regression analysis of the gathered interpreted data found that equation six gave the most suitable correlation:

$$y = -0.7608x + 6.436 \quad \text{(equation eight)}$$

In equation eight x is expressed as the time lapse in days and y is the predicted population expressed as the log base 10 and is shown as Table 3.6.1.

Table 3.6.1 Relationship between time lapses (days) and predicted population (pac/ml) of APB

	Time Lapse (Days)			
	1	**2**	**3**	**4**
High population	2,290,000	397,000	68,900	11,900
Average population	**473,000**	**82,100**	**14,200**	**2,470**
Low population	97,800	16,900	2,940	510
	5	**6**	**7**	**8**
High population	2,070	359	62	10
Average population	**428**	**74**	**12**	**2**
Low population	88	15	2	1

3.7. Denitrifying Bacteria (DN- BART)

– Grey Cap

Parameter code: nitrite biotester

3.7.1 Introduction

Essentially all of the atmospheric gaseous nitrogen (N_2) has been derived from the process of denitrification has been driven primarily by the denitrifying bacteria (DN is short) for denitrification which primarily involves the reduction of nitrate. This activity is extremely important not only to the environment but also in geochemical terms as a major factor in the stabilization of the atmosphere with a dominance of nitrogen along with a significant presence of oxygen. It is therefore an extremely important stage in the nitrogen cycle in the crust interchange with the atmosphere on 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, 3.8.1.) and is finally reduced back to nitrogen gas by denitrification which is controlled by the denitrifying (DN) bacteria. DN bacterial communities are one of the two bacteriologically influenced events that add gaseous nitrogen to the atmosphere. Balancing this nitrogen supplementation are the nitrogen fixing bacteria that return the nitrogen to various fixed organic and inorganic nitrogenous compounds. When organic nitrogen is degraded reductively then ammonium is one of the principal nitrogenous products. Completing the nitrogen cycle the nitrifying (N-) communities oxidise the ammonium to nitrate. When reductive conditions occur then this nitrate daughter product is degraded eventually to nitrogen gas via nitrite by the DN-bacterial communities. 3.7.1, introduction deals with the DN community.

Denitrifiers reduce nitrate through to nitrite and then 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. If such waters become anaerobic (free of oxygen) and relatively rich in organic matter then conditions exists for DN communities to bloom and generate nitrite and then nitrogen gas. .

A common use for the presence of aggressive denitrifying bacteria in waters is that these bacteria signal the later stages in the degradation of nitrogen-rich sewage and septic wastewaters.

Aggressive presence of denitrifiers in water can be used to indicate there is a potential for the water to have become 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. Generally the denitrifiers follow the presence of coliform bacteria in dominating the reductively fermenting sewage and septic wastes.

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 functionally active and that the conditions in the soil are reductive. 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:

NO_3^- -------→ NO_2^- --------→ NO ---------→ N_2O ---------→ N_2 gas

Nitrate Nitrite Nitric oxide Nitrous oxide Nitrogen

(1) (2) (3) (4)

Bacteria involved in DN communities are commonly not able to perform all four steps (e.g. 1, + 2 + 3 +4) in the denitrification process and have been divided into four distinctive communal group 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)

One of the largest groups of denitrifying bacteria is the enteric bacteria which includes many of the coliform bacteria. All of these bacteria listed below perform denitrification functions under anaerobic (oxygen-free) conditions in a reductive environment. The range of genera associated with DN communal activities includes: *Actinomyces; Aeromonas; Agrobacterium; Alcaligenes; Arthrobacter; Bacillus; Bacteroides; Campylobacter; Cellulomonas; Chromobacterium;*

Citrobacter; Clostridium; Enterobacter; Erwinia; Escherichia; Eubacterium; Flavobacterium; Geodermatophilus Halobacterium; Halococcus; Hyphomicrobium; Klebsiella; Leptothrix; Micrococcus; Moraxella; Mycobacterium; Nocardia; Peptococcus; Photobacterium; Proteus; Pseudomonas; Rhizobium; Salmonella; Serratia; Shigella; Spirillum; Staphylococcus; Streptomyces; Thiobacillus; and *Vibrio.*

As can be seen from the above list, it consists of very wide ranging genera of bacteria each capable of denitrification. Their ability to perform denitrification is controlled, in part, by the availability of the nitrate, nitrite, nitrous and nitric oxide substrates. The selective culture medium contains peptone and nitrate along with important macro-nutrients. Each sterile DN-tester pellet has a dried weight per tester of 247.5±1.5mg.

3.7.2. Reaction patterns for the DN- Tester

Denitrification is recognizable in the DN- tester when the various inorganic forms of nitrogen dominated by nitrate are reduced through to nitrogen gas. This gas then collects as gas bubbles forming into a foam ring around the ball. This is the only reaction recognised for the DN- tester.

FO - Foam ring around floating ball

There is only one reaction recognized ion the DN- tester that occurs only when the nitrate is completely denitrified to nitrogen gas which then collects as a detectable continuous foam of interconnected gas bubbles forming a ring around the ball. Casual presence of gas bubbles attached to the side walls or on the underside of the ball should be ignored since these bubbles relate to the transient presence of either gases such as carbon dioxide or oxygen. The formation of an interconnected ring of bubbles around the ball is the only indication that an FO reaction has occurred.

In practise the culturing sample in the DN- tester usually first goes cloudy (turbid) but this should be ignored as an insignificant reaction. It is only when there has been the observed presence of very many interconnected gas bubbles forming a ring of foam around the ball that an FO reaction can be declared This shows that complete denitrification has occurred and the

denitrifying bacteria are present.

It should be noted that the foam (FO) ring may consist of as little as a single layered ring of bubbles to thick multilayered foam that can be as much as 5mm thick. Commonly this FO ring originating at the equator of the ball and extends upwards. Thick foam commonly will last for one to two days and, on rare occasions. Often this foam ring will support distinctive bacterial biomass that can even grow above the foam (e.g. iron oxidizing bacteria producing ferric-rich plates).

3.7.3. Bacterial activities recognized by denitrification in the DN- tester

Three ATCC strains of bacteria are used to confirm denitrification in the DN-tester through the production of an FO reaction. These species include: (13048) *Enterobacter aerogenes*; (19606)* *Acinetobacter calcoaceticus*; (27853) *Pseudomonas aeruginosa* and (25922) *Escherichia coli*. All three species give the foam ring after two to three days of incubation at room temperature or **(**28±1 ^{o}C). One of the three species (*) does not exhibit clouding while the other two do exhibit clouding which usually precedes the formation of the FO ring. Quality management can also be performed using secondary effluent from a aerobic municipal sanitary wastewater treatment plant.

3.7.4. Time lapse determination of DN- predictive active cell populations

Populations were assessed using serial dilutions of the samples to 10^{-9} using sterile Ringers as the diluant and 1.5ml as the transferred volume between diluents. All 15ml of each completed diluant was then transferred to DN- testers which were then incubated for ten days at room temperature (22±2 ^{o}C) and observed for the time lapse to the generation of a FO reaction. For the greater the dilution then there was an extension in the time lapse with no reaction being observed in ten days of incubation taken to mean no DN activities were detected. Confirmation was obtained by subculture 1.5mL of positive FO into a fresh DN- tester and incubating to confirm the occurrence of the FO reaction. Colony forming units were recorded by spread plate analysis using 0.1mL of each diluant on R2A agar with incubation for ten days at room temperature. Regression analysis of the data found that equation nine gave the most suitable correlation:

$$y = -0.930x + 7.19 \quad \text{(equation nine)}$$

In equation nine x is expressed as the time lapse in days and y is the predicted population expressed as the log base 10 given in Table 3.6.4.1.

Table 3.7.1 Relationship between time lapses (days) and predicted population (pac/mL) of DN.

	Time Lapse (Days)			
	1	**2**	**3**	**4**
High population	2,290,000	397,000	68,900	11,900
Average population	**473,000**	**82,100**	**14,200**	**2,470**
Low population	97,800	16,900	2,940	510
Days:	**5**	**6**	**7**	**8**
High population	2,070	359	62	10
Average population	**428**	**74**	**12**	**2**
Low population	88	15	2	1

3.8 Nitrifying Bacteria (N- Bart) – White Cap

Parameter code: nitrate biotester

3.8.1 Introduction

Nitrogen is the dominant gas in the atmosphere and is dynamically affected by the activities of three bacterial communities in which the denitrifiers (DN) move nitrogen into the atmosphere by reducing nitrates and nitrites; nitrogen fixers that convert nitrogen gas into biomass both aerobically and anaerobically, and the nitrifiers (N) that oxidise ammonium (common reduced form of nitrogen) to nitrate. Nitrification serves as the major route by which ammonium is aerobically oxidized to nitrate. There are two stages in the nitrification process:

$$\underset{\text{Ammonium}}{NH_4^+} \xrightarrow{(1)} \underset{\text{Nitrite}}{NO_2^-} \xrightarrow{(2)} \underset{\text{Nitrate}}{NO_3}$$

Nitrifying bacteria are divided into two groups according to which of the above reactions they are able to perform:

Group 1 -stage (1) only- Nitrosofiers -*Nitrosomonas*

Group 2-stage (2) only - Nitrifiers -*Nitrobacter*

There are complimentary relationships between the nitrifying and the denitrifying bacteria on the bio-generation of atmospheric nitrogen. Here DN communities add nitrogen to the atmosphere and the N communities oxidise ammonium to nitrate as an essential stepping stone to denitrification. The other direction in which nitrogen is entrapped in biomass is in part a function of the nitrogen fixing bacteria. In simple terms oxidative conditions trigger nitrification with nitrate as the product and reductive conditions will favour denitrification with nitrogen gas as the end product.

Problems arise when testing natural samples of water or soil since both groups are commonly present either producing or utilizing nitrite respectively. Nitrate has been to be transient as a final product and in natural samples rapidly become reduced to nitrite. In developing an N- tester

system for nitrifying bacteria in natural samples, the terminal product of a positive determination (nitrate) was therefore found to be unreliable. This lack of precision was due to the common 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-tester restricts itself to detecting the nitrosofiers (group 1) that generate nitrite. This nitrite will be oxidized to nitrate by the nitrifiers (group 2) only to reappear again when reduced back to nitrite when any intrinsic denitrification activity occurs in the culturing sample of 7.5ml. This smaller volume is used since the tester is designed to maximise oxidative conditions (e.g. terse is laid on its side with three balls to increase internal surface areas.

Nitrifying bacteria are an important indicator group for the oxidative recycling of organic nitrogenous materials from ammonium (the end point for the reductive decomposition of proteins) to the production of nitrates. In waters, the presence of an aggressive population of nitrifiers is taken to indicate that there is a potential for significant amounts of nitrate to be generated in waters particularly under aerobic (oxidative) conditions. 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 soil. Nitrate is a highly mobile ion in the soil and will move (diffuse) relatively quickly while ammonium tends to remain 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. Active presence of nitrifying bacteria in water can be used to indicate the potential for waters to have been polluted by nitrogen-rich organics from such sources as compromised septic tanks, sewage systems, industrial and hazardous waste sites. Nitrification and denitrification are essentially opposing processes that function in reverse sequences to each other. It is recommended that, where activity is determined (i.e. nitrate detected) then such waters should be subjected to further evaluation as a hygiene risk through a subsequent determination for the presence of coliforms. 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 reductively from the denitrification of nitrate may also be oxidized back again to nitrate. This means that cycling between nitrite and nitrate can occur around oxidation-reduction potential interfaces.

This test detects the nitrifying bacterial communities that are able to oxidize ammonium (NH_4) to nitrite (NO_2) and on to nitrate (NO_3). This test uses a selective medium for the bacteria able to specifically oxidize ammonium to nitrite by examining chemically for the nitrite product (i.e. group 1). The additional two balls used in this inner tester provide a larger solid: medium: air area on the upper hemispheres of the three balls. Here the tester is laid on its side so that all three balls are exposed above the 7.5ml sample. This encourages nitrification in the liquid film over the balls. In the early stages, the first (product) nitrite is detected at these sites. A reactant cap used in the test specifically detects the presence of nitrite that is generated during the early stages of nitrification. If the sample being tested also contains denitrifying bacteria, nitrite may again be created by the reduction of nitrate (denitrification). This test method has been developed in consideration of the greater likelihood of nitrite being detectable rather than the (product) nitrate. Note that this test cannot function in water samples with a natural nitrite level of greater than 3.0ppm. Water samples with greater than 28ppm of nitrite will automatically turn the liquid medium to a yellow color when the reaction cap test is applied.

In the N- tester there is a total dried active chemical component with the pellet and in the reaction cap of 209±3.0mg per tester. The selective culture of nitrifiers has been based on ammonium sulfate mineral salts and the reaction cap specifically is used to detect the presence of nitrite after the standard five day incubation period.

3.8.2 Reaction patterns for the N- tester

This tester is an unusual tester in that the presence of nitrifying bacteria is detected oxidatively after a fixed incubation period of five days at room temperature. Positive detection is therefore achieved based on the presence (+) or absence (-) of nitrite. Nitrification involves the oxidation of ammonium to nitrate via nitrite. Unfortunately, in natural samples, there are commonly denitrifying bacteria present and these can reverse the reaction by reducing the nitrate back to

nitrite. If denitrification is completed then this nitrite may be reduced further to nitrogen gas (under reductive 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 and denitrification is minimalised. The reagent administered in the reaction cap detects nitrite specifically by a red color reaction. To conduct this test at five days then the tester is turned upright and the cap removed and replaced with the reaction cap. The charged tester is now inverted onto the cap and left for three minutes to allow the reactants to dissolve. After the reactants in the cap have dissolved (less than three minutes) then the tester is inverted again (cap side up) and left for three hours for any reaction to fully mature. Reactions are interpreted by the amount of pink-red coloration generated both on the balls and in solution. There are three recognized levels of positive detection of nitrite as an indicator of nitrification (Table 3.8.1.).

Table 3.8.1 Bacterial consorms recognized by reaction signatures in the N- tester

Reaction	Color balls	Color solution	Pantone color on ball	Pantone color in solution
PP, partial pink	Partial pink*	Clear-yellow	514 to 515	0/7404CS
RP, red pink	Red deposits**	Pink	7425 CS	706 CS
DR, dark red	Dark red	Pink	7426 CS	708 CS

Note: * partial pink means that the three balls are coated with pink over only a part of the area that is commonly the region exposed to air during the test; ** red deposits occur all over the balls but are particulate in nature and erratic in occurrence; the reaction represents the population size of the nitrifying population in the sample and does not reflect the variety of microorganisms that may have been present in the sample:

(PP) relatively inactive population of nitrifiers ($< 10^2$ nitrifiers/mL) associated with aerobic slime forming bacteria in a consortium;

(RP) moderately active population of nitrifiers ($> 10^2$ and $< 10^5$ nitrifiers/mL) forming significant part of the bacterial flora; and

(DR) indicates a dominant population of nitrifiers ($> 10^5$ nitrifiers/mL) in the sample.

3.8.3 Bacterial consorms recognized by reaction signatures in the N- BART

There is two A.T.C.C. strains 25391 *Nitrosomonas winogradski* and 19718 *Nitrosomonas europae* that can be used to validate the N- tester through giving one of the positive reactions (PP, RP or DR) to the nitrification of ammonium to nitrite.

3.8.4 Determination of predictive active nitrifying populations

The notes relating to Table 3.7.2.1 do allow a semi-quantitative prediction of the active nitrifying bacterial population. However in the event there needs to be greater precision in the prediction of the active nitrifying bacterial population. This can be achieved when there is serial dilution of the liquid sample. This method employs 10mL sterile distilled water to form a dilution series from the original liquid sample. Here 1ml of original sample is added to 10 mL of the sterile distilled water and mixed to make a tenfold dilution. Withdrawing 1ml from that dilution and adding to a second 10ml of sterile distilled water now creates a further dilution of one hundredfold from the original sample. This can be repeated to create additional dilutions of the original sample (e.g. thousand fold, ten thousand fold etc). To determine the presence of nitrifying bacteria then each 10ml dilution can be used to determine whether there is activity by incubating for five days in separate N- BART testers following the protocol defined in the "Certificate of Analysis". This can then be used to determine at which dilution there is no longer evidence of nitrification (e.g. no coloration as described in table 3.7.2.1). For example if evidence was found for the presence of active nitrifiers at 10^{-3} (thousandfold or three orders of magnitude but none at 10^{-4} (ten thousandfold or four orders of magnitude) dilutions then that would mean that were greater than 1,000 but less than 10,000 active nitrifiers per mL in the original sample being tested. By undertaking duplicate or triplicate analysis of wastewater samples and undertaking dilution series down to 10^{-4} then it would be possible to semi-quantitatively monitor the activities of nitrifying bacteria using the N- tester.

3.9. Micro-algae (ALGE- BART) – Dark Green Cap

Parameter code: microalgae biotester

3.9.1 Introduction

Green photosynthesising microbes commonly called algae or more precisely micro-algae are extremely abundant not just in green blooming waters but also in soils. In soils the depths where the soil algae grow is limited by light penetration into the soil. This occurrence means that these green blooms in soil are not obvious and so are generally ignored unless the green biomass emerges out of the soil. The designation of ALGE- tester examines for the presence of active micro-algae in the sample. These plant-like microorganisms are able to photosynthesize using light as the energy source for growth. This range can include grass green micro-algae (*Chlorophyceae*), blue-green algae (*Cyanobacteria*), desmids, diatoms and Euglenoids.). The tester does not detect the anaerobic photosynthesising sulfur (e.g. *Chromatium*) and non-sulfur bacteria (e.g. *Rhodopseudomonas*) since these require very specialised reductive conditions. ALGE- tester is distinctly different from the other tester products because it is designed to recover and culture photosynthesizing microorganisms that utilize light and releases oxygen as a product at the same time.

ALGE- testers use a modified Bold's culture medium which does not contain organics beyond trace levels but does contain the basic inorganic nutrients for plant growth (nitrogen, phosphorus, potassium, sulfur etc.). Carbon is presented in the tester as bicarbonates and the medium is made slightly alkaline (pH, 8.2) to encourage the micro-algae to utilize this form of carbon. One major problem with the ALGE- tester is that the growth of micro-algae tends to be slow and require the presence of light (for photosynthesis). Most micro-algae can actually utilize very low levels of light for growth. For example the charged ALGE- tester can be placed on its side and set about 60cms from a single 40 watt daylight fluorescent light and grow. Other differences in the ALGE-tester relates to the micro-algae growing within the pores of the various woven materials layered around a part of the tester. Some micro-algae gravitate towards the semi-saturated material above the culturing sample within the tester while others grow within the saturated textile material or within the liquid medium itself. In practice, this test takes a maximum of 32 days to detect significant populations of micro-algae. In practise the ALGE-tester can be used as a simple

presence/absence (P/A) tester when read at 32 days but the tester is capable of indicating to some extent the population sizes and the types of micro-algae present in the water sample to be semi-quantitative. Twice weekly observations for 32 days (e.g. at 4, 8, 12, 16, 20, 24, 28 and 32) should be undertaken to observe the various forms of algal growth. Note that table 3.8.4.1 does give a link between population size and time lapse observed.

Different algae utilize different sites in the tester because of the two woven materials and the lateral position of the tester lying on its side within an outer vial (bottle). These form into six possible reaction patterns in the test sample (see Section 3.8.2). Observations can determine:

(1) Levels of activity of the micro-algae (activity) through the time lapse to when the reaction is first observed; and
(2) Community composition of the active micro-algae present in the sample.

By the routine (e.g., monthly) testing of the environment (water, soil or wastewater) using this technique, the levels of activity can be determined and monitored To conduct the ALGE- test, it is necessary to add 15 mL of the water sample to the inner ALGE-tester. Once this has been done and the inner test vial returned to the outer test vial that is then capped, the test can begin. To initiate growth, it is recommended that the tester be laid on its side under a light source. Continuous light is preferred. Incubation is at room temperature and the tester protected from any excessive heating due to any artificial lights that are used. Note that temperatures in excess of 80^{o}F (30^{o}C) may inhibit algal activity. Under no circumstances should the test be severely agitated or shaken during the test period. To observe the test, gently examine the tester for the presence of colored patches (often green initially). If the test is negative, the woven material should remain white and there should be no colored patches or cloudiness in the water medium. A positive reaction may be recognized when there is either: a colored cloudiness, a distinctive patch of color or a colored deposit generated within the tester. Low magnification stereo microscopy can be used to directly observe the types of algae growing in the tester.

If there is a need to determine the micro-algae population in a soil or semi-solid slurry then technique would need to be modified. This is necessary to reduce the potential detrimental effects that could be caused by a high organic nutrient in the soil. Such loadings could stimulate the growth of heterotrophic microorganisms at the expense of the micro-algae. To correct for this, take 1 g of the soil or slurry and suspend it in 14 ml of sterile distilled water. Agitate for one minute to disperse the particles evenly into suspension and also break up some of the larger structures. Aseptically withdraw a 1ml sample from the midpoint of the suspension and transfer to 14 ml of sterile distilled water. Use this 15mL suspension to charge the ALGE-tester and follow the standard procedures. Because the micro-algae tend to grow slowly, the generation of a growth may be difficult to determine initially. Many micro-algae may initially start to grow generating a green color since the chlorophyll pigments used for photosynthesis are often dominant at that time. But as the growth continues, other pigments such as the xanthophylls may become dominant and change the color of the growth. This color shift may involve several different colors dominating over time (e.g., green to yellow to orange to brown).There are several habitats presented in this tester which can encourage the growth of different micro-algae. These habitats include semi-saturated porous, saturated porous, aquatic; liquid: solid and liquid: air interfaces. Nutrients provided are inorganic nutrients commonly used by the micro-algae that, together with constant illumination, provide a preferential habitat for these microbes. Growth is slower because of the longer generation times commonly found in the micro-algae. The ALGE-tester includes Bold's cultural medium pellet (Mineral salts, EDTA, with macro- and micro-nutrients) along with the cellulosic and polypropylene textiles which have a combined dried weight per tester of 2,540±20.0mg.

3.9.2. Reaction patterns for the ALGE- Tester

There are therefore six reactions that can be observed in the ALGE- BART tester as shown in Table 3.9.1. Colors are defined by Pantone colors. These would be subject to individual judgements and the numbers shown represent reasonably close fits to the defined colors.

Table 3.9. 1 Six Reaction commonly observed in the ALGE- tester

Code	Growth	Description	Pantone
GG	**Grass green**	A grass green growth seen through the porous textile and usually concentrated at or below the water line. As growth matures then flocculent greens may be observed in the liquid.	555
FG	**Fuzzy green patches**	Reactions commonly occur here above the water line in the semi-saturated textiles. There develops intense patches of green growth that then has an ill-defined edge.	555
OB	**Red, orange, brown patches**	Red, orange or brown patches are formed in the textile above or at the water line. These growth change shade (orange to red to brown) as the growths mature.	7425 715 7524 7525
YB	**Yellow beige patches**	Poorly defined light yellow to beige patches of growth occur on the textile at small sites that are difficult to spot without a lower power reflective microscope.	7404 728
GF	**Green flocculent**	Grass-green flocculent deposits grow in the floor of the tester and may extend up into the textile fabric below the water line.	554 555
DG	**Dark green to black patches**	Recognized as dark green to black growths centered in the textile at the water line in the tester. This is commonly a secondary reaction after GG, FG or GF.	582 567 5535

Note: the colors are shown as Pantone equivalences to the Red. Blue, Green (RBG)

In summary, the colors present by the six different reactions do occur at different sites in the ALGE- tester. These are defined as: (GG), green growth at or above the water level; (FG), irregular patches of green growth over the woven material; (OB), patches of red, orange or brown growths below water level; (YB), yellow patches diffuse over the woven material; (GF), green deposits and/or green growth in the woven material; and (DG), blue-green or black growth commonly at the water level.

3.9.3. Micro-algal communities recognized by reaction signatures in the ALGE- tester.

There are some micro-algal communities that do present, in sequence, more than one reaction type. Common reaction pattern signatures (RPS) are:

(G – DG) Cyanobacteria present with possible *Nostoc* dominance;

(FG – DG) Grass-green algae with cyanobacteria present;

(FG – OB) Grass-green algae maturing;

(YB – OB) Diatoms or desmids may be dominant;

(GG – GF) Grass-green algae maturing without pigment production;

(GG - GF – DG) Grass-green algae are dominant but with cyanobacteria eventually dominating.

It should be noted that the RPS signature displays the reaction patterns in the order that they were observed. For example, GG - GF - DG signature indicates the order for the reactions observed were firstly, GG; secondly, GF; and thirdly, DG. The signature obtained from an individual water sample will provide an initial understanding of the type of algal community present in the water sample.

3.9.4. Time lapse determination of predictive active micro-algal cell populations

ALGE- tester is a semi quantitative measure of the numbers of micro-algae in the sample is created by various algal communities that may be present. Population determinations has primarily been by microscopic identification and enumeration on 0.45micron filters through which small aliquots of the sample in question have been filtered using 20±1psi to create suction. Using this data base then a linear regression analysis of the pooled data found that equation ten gave the most suitable correlation:

$$y = -0.1638x + 5.515 \quad \text{(equation ten)}$$

In equation one x is expressed as the time lapse in days and y is the predicted population expressed as the log base 10. (Table 3.9.2)

Table 3.9.2 Relationship between time lapse (days) and predicted population (pac/ml) of micro-algae using the ALGE- Tester

	Time Lapse (Days)			
	1	**4**	**8**	**12**
High population	315,000	101,000	22,400	4,970
Average population	**224,000**	**72,400**	**16,000**	**3,540**
Low population	159,000	51,500	11,400	2,540
	16	**24**	**32**	
High population	1,100	53	2	
Average population	**783**	**38**	**1**	
Low population	558	27	1	

3.9 Fluorescent Bacteria (FLOR - BART)

- Yellow cap

Parameter code; fluorescing biotester

3.10.1 Introduction

Each FLOR- tester employs a selective chemical nutrient pellet which has a dried weight of 339.6±1.5mg per tester. The culture medium is rich in proteose and peptone-tryptone and these chemicals trigger the development of fluorescent pigments during the test. Fluorescing bacteria are defined as:

> "Any bacteria that generate water-bonding polymers outside of the individual cells which take on the form of a coherent slime within which there are generated Ultra-Violet light fluorescing pigments that impart distinct colors to at least a part of the growing biomass"

There are two groups of fluorescing pigments that are significant in the FLOR- tester. These generate pigments that glow under ultra-violet light when the tester is illuminated. When the pigments are very intense then the glow is also visible in sun- or room- light. In general any UV light will trigger the fluorescence that may be dominated by either an Pale Blue (PB) or a greenish yellow (GY) color. In general the PB fluorescence tends to be limited to the top quarter of the tester for a few days while the GY tends to last longer and be present down half the tester below the ball. PB occurs when *Pseudomonas aeruginosa* is a dominant member of the bacterial flora in the sample and generates a pale blue (PB) glowing reaction in the top quarter to one third of the tester and usually lasts between three to five days and then commonly fades away. GY occurs when the *Pseudomonas fluorescens* species group is significantly present in the sample leading to a glowing greenish yellow (GY) reaction that extends downwards one third to a half into the tester. The glowing lasts commonly from five to ten days and, on some occasions, does

not fade away. PB reaction is significant because the most likely cause would be *Pseudomonas aeruginosa* that has been linked to another of nosocomial bacterial infections ranging from lung to skin infections. These can be a particular risk to those with impaired immunity. For the GY reaction which is triggered by the *Pseudomonas fluorescens* species group the health risk is much lower and these types of bacteria are commonly associated with conditions where there are intense bacterial activities occurring (such as at bioremediation sites and oxidative sites where organics are being degraded).

3.11 POOL BACTERIA (POOL- BART)
- LIGHT BLUE CAP

Parameter code: pool biotester

3.11.1 Introduction

POOL- BART tester was a specialty test for bacteria known to be active in swimming pools and hot tubs. Swimming pools and hot tubs are common recreational means for relaxing in a warm water environment. Unfortunately such warm water environments can also be breeding grounds for bacteria both within the pool or tub water and in the filters, pumps and heaters often associated with the facility. POOL- tester examines the levels of bacteria present in the water sample that could be linked to either biofouling or some type of hygienic hazard risk. Even when the tub or pool water appears to be clear that does not mean that the water does not have significant bacterial loadings. This is because the level of bacterial activity may not be directly visible or that the infesting biomass is growing out of sight within the filters, pumps and heaters. Generally the use of biocides (such as chlorine-based bleaches) will retard the bacterial activity within the pool or tub but have a lesser effect on the waters passing through the equipment. As a direct result of this then the bacterial risks become out of mind and out of mind. These risks are exaggerated by intensity with which the tub or pool is used. Facilities subjected to greater use (i.e. more body surface area to available tub or pool water volume) inevitably will place a greater demand on the filters, pumps and heaters and increase the potential for fouling. Pool related bacteria tend to be those that function more effectively in warmer waters, the POOL- tester has been developed to examine for:

> "Bacteria that generate water-bonding polymers to the form coherent slimes and/or thread-like structures within pools, hot tubs and in associated equipment. These bacteria can cause clouding of the waters, extensive and potentially dangerous slime on the walls and can also reduce efficiencies in associated filters, pumps and heaters. Some of these bacteria could be nosocomial pathogens and present hygiene risks to the users of the facility"

To achieve sensitivity the culture medium used in the POOL- tester has a reduced nutrient loading of 169.8±1mg/tester pellet. The culture medium used is a broad spectrum type that would trigger the growth of the broad range slime forming bacteria. For this purpose the medium is dominated by peptones. Relationship between time lapse in days and the predicted population (in pac/mL) are given in Table 3.10. 1.

Table 3.11.1, Daily Observations of the POOL- tester showing variability in Predicted Populations (pac/mL)

	Time Lapse (days)			
	1	**2**	**3**	**4**
High population	1,730,000,000	400,000	48,000	26,500
Average population	**600,000**	**139,000**	**30,000**	**13,000**
Low population	399,000	50,000	11,630	3,000
	5	**6**		
High population	11,000	2,160		
Average population	**2,550**	**500**		
Low population	588	115		

In the event that the POOL- tester does detect fluorescence using a UV light then concern should be given to the hygiene risk if the color is pale blue. Risk from the detected bacterial populations are defined in Table 3.10.1 where red would indicate that a significant hygiene risk existed, yellow would mean borderline and green would indicate no significant risk. It has to be remembered that the method of sampling is critical here to expose the potential risks from fouled filters, pumps and heaters.

3.12 Enhanced bacterial activity (ENH- BART)

- Flexible beige cap

Parameter code: bioenhancer

3.12.1 Introduction

ENH- Bart is a biological enhancing system designed to maximally increase the bacteriological metabolic activity in bacterial cells. Focussed, for this activity, is placed on the ability of cells under test to maximise their production of high energy storage. This energy storage take a form common to all living cells primarily using adenosine triphosphate (ATP) as the prime marker of the level of energy storage. However second generation ATP biochemical test methods rely on the direct measurement of the level of ATP at the moment that the test is performed. When the ENH- enhancer is used then the level of activity is stimulated to achieve a better precision. Stimulation involves placing the sample in the ENH- enhancer which is immediately inverted six times in one minute. This simple act (rotation at 3rpm or 6 manual inversions for one minute total) now presents the bacteria in the sample with supersaturation with oxygen and a flood of rapidly dissolving chemical nutrients. The net effect of this one minute of radical stimulation (primarily with an oxygen and nutrient flood) is that there is a stimulation of ATP production ranging from x3 to x10. At the same time it has been found that the precision improves dramatically. Consequently the ENH- enhancer has become the first cultural method to test samples to determine whether there is indeed enough bacterial activity to warrant the second level testing using selected Bart tester. This ENH bioenhancer is defined as:

> "Rapid means of generating more precise increases in ATP activity within a test sample. Here it is the total ATP (tATP) that is determined after one minute of exposure within the ENH- enhancer. These exposure conditions are critical and involve rotating the charged enhancer three full rotations or inversions in one minute and then taking a mid-point sample for tATP determination. The data generated is referred to as the enhanced tATP or E-tATP and is commonly in picogram/mL. In practise it has been found that the E-tATP has better

precision and is three to ten times higher than the straight total ATP”

This technology has been developed for applications in the oxidative municipal wastewater treatment plants and it has proven to generate better precision (commonly 2 to 5% variance) than conducting straight tATP tests on the unenhanced sample where the variance ranges between 8 and 15%. E-tATP technology has been found adaptable to a broad range of samples and still maintain acceptable precision. For samples containing salt within brackish waters, salt solutions and saturated salts then the method can still be applied but using the QGA (Luminultra technologies Inc, Canada) to filter the cells from the sample (after enhancement), wash them and then perform the tATP determination. Chapter 14 gives the detailed protocol for performing both the straight tATP and then the QGA method for brackish and salt-rich waters.

3.13 Standard Bacteriological Community Code (SBCC)

3.13.1 Introduction

There are ten different Bart testers that can be included in a determination of the nature of the bacterial community. To achieve this standardised recognition of the various communities the primary approach is to employ the DBI-standardised bacteriological community code (SBCC) which is distinctive for each type of Bart tester that has been used. The SBCC has a standard seven digit format recognised as XXYYYYZ in which XX is a two letter designation of the Bart tester type shown in shown in Table 3.13.1.

Table 3.13.1 Conversion table to allow the two letter recognition of Bart tester type

Bart type	XX code
IRB-	IR
SRB-	SR
HAB-	HA
SLYM-	SL
APB-	AP
DN-	DN
N-	NO
ALGE-	LG
FLOR-	FL
POOL-	PO

Note: N- is expanded with the letter "O" to clarify that the daughter product would be nitrate (NO_3)

YYYY are the first four reactions coded into a numeric sequence; and Z relates environment from which the community was sampled. Z relates primarily to the oxidation reduction potential (ORP) as being oxidative (as C if carbonates are oxidatively deposited as white crystals in the base of the tester; or as X if no carbonate deposition is observed), interfacial midpoint of the ORP shown as I; and then reductive shown as an R but in the event there is a blackening (due to

the presence of sulfides or elemental carbon or hydrocarbons) which is signified as B. Tester type is and forms the first two letters in the SBCC.

The four Y letters are used as numerical representations of the various recognised reactions. Conversion table for taking the two letter descriptor for each reaction (listed on all certificates of analysis) and the Y value is shown in Table 3.12.2.

Table 3.13.2 Numeric conversions of the two letter reaction code to a single number numeric code for the Y values in the BSCC.

	Designated Y numeric value for reaction code							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
IR	FO	CL	BC	BG	BR	GC	RC	
SR	BT	BB	BA					
HA	UP	DO						
SL	CL	DS	CP	SR	TH	PB	GY	FO
AP	DY	DYB						
DN	FO							
NO	PP	RP	DR					
LG	GG	FG	OB	YB	GF	DG		
FL	CL	PB	GY					
PO	CL							

The last letter in the BSCC (Z) relates to the ORP value in the environment with carbonate (C) deposition being considered oxidative, as well a dominant respiratory activities that are oxidative (X), growth and activities at the ORP interface (I) occurs between oxidative and reductive conditions, and reductive activities (primarily fermentative) are show as R while some conditions cause the reduction to generate black products (B) as either sulfides or reduced forms of organic carbon (e.g. petroleum hydrocarbons, elemental carbon).

The trigger events that can be used to determine whether the sample warrants further investigation is given in section 3.12.2. Interpretation of the BSCC can be done in section 3.12.3 which employs the principal codes that have been experienced in past Bart testing. As an

example the BSCC code generated of IR2350C would mean iron related bacteria that are iron oxidizing, slime forming and oxidizing to carbonates.

3.13.2 Trigger protocols to determine whether full BSCC should be attempted.

Bacterial communities exist in a complex interaction in which the law simply results to the survival of the fittest and elimination of the non-contributors. Now a non-contributor may be either become feedstock for the growing community, or simply shunt into a dormancy state where the communities simply ignore their presence. In any given environment there may be more than one community functioning within that environment within unique parts of that sample's habitat. At the sample level then these communities appear dispersed but within the different Bart testers the selective conditions encourage the growth of part of that community that relate to the conditions generated within the Bart tester. In practise therefore an environment may contain a number of different bacterial communities that essentially operate separately within the micro-cosms created by habitats dominating the sample's original environment. In general in an active sampled environment there could commonly be between six and thirty two different bacterial strains that are active while an indeterminate number are not active. Active bacterial strains within that community are in a state of harmony within the micro-cosm and would dominate. It would be expected that these bacteria would dominate many aspects of the functioning micro-cosm. Further these bacteria would dominate the micro-cosm when suitable cultural identification protocols are presented. For the various Bart testers there is automatically a selective cultural bias to determine whether these particular bacteria are present that could be related to one specific defined community. Dormant bacteria that are in some form of survival mode (e.g. ultramicrocell, endospore, and exospore) may not be active within the sample's microcosms but could become active in the cultured environments in the Bart testers. Essentially there are two groups of bacteria; the active (functioning in harmony with the other active bacteria); and the stressed (basically dormant and not detected by the active bacteria.

Trigger protocols have to examine the levels of microbiological activity that are detectable in the sample's micro-cosms. There are three stages in triggering an understanding of the bacterial community: **(1) determination of the basic metabolic activity** in the sample; **(2) differentiating the potential communal activity** that could occur in the sample; and **(3) identify the communal structure** in the sample. All three stages must be conducted using the original sample. Present practices for identification commonly involve initially isolation of

bacterial colonies grown on some specific agar culture medium. Such practices are totally unsuitable for the triggered identification of bacterial communities. This is because such agar-selective practices eliminate the vast majority of the bacteria that do not have the ability to be effectively being cultured on such agar media. Colonies that are cultured represent but a small fraction of the bacterial types that are present and active in the community. For these obvious reasons agar culture does not form a part of the understanding of the bacterial communities. Traditionally based agar selective practices have only a limited scientific capability where the desire is to culture specific strains of bacteria that are known to be culturable on agar based.

Determination of metabolic activity is based upon the determination of the enhanced total adenosine triphosphate (E-tATP) that is addressed comprehensively in Chapter fourteen. Here the object is to determine at that moment of testing the sample the actual level of E-tATP activity. This reflects the levels of metabolic activity that is occurring at the time of testing and enhancement. Enhancement is a sixty second cultural stimulation which causes the E-tATP to elevate at between x3 to x10 but have considerably better precision. Here the enhancement forms a cultural stimulation that causes ATP activity to increase in proportion to the number of potentially active cells. Data generated is presented in picograms (10^{-12}) per mL or gram of sample. This level of determined E-tATP activity can range from 0 to 10^{7}pg/mL or g. In practise the generated data can be interpreted to determine the nature of the bacterial activity within the sample (Table 3.11.3). It should be recognized that a zero (0.0 pg/mL) does not mean that the sample is sterile but that there is no ATP-based activity occurring in that sample at that moment in time. It does not mean that there are no dormant survivors and it could mean that the community, if culturally detected, is dominated by survivors that not actively metabolising cells. In differentiating the potential community activity it is the Bart testers employed in the nest stage that can now determine the potential for communal bacterial activity in the sample in question.

Bart testers have, by their nature, much greater abilities to differentiate the sample's global population into fractions that will, or will not grow under the conditions presented in the Bart tester. It should be remembered that in the act of Bart culturing the sample there remains a possibility that competition between the various strains and communities may cause some of these to become suppressed or dominate some of the other strains. Bart culturable differentiation of the bacterial communities within the sample therefore generates a very different data set than the E-tATP which forms a simple expression of the enhanced potential total ATP activity. E-

tATP generates a single numeric interpretation of the state of the biological activity within that sample. It does allow a quantitative evaluation of the potential for active bacteriological activity within the sample. Bart testing is both a quantitative and qualitative differentiation of the bacterial communities. Quantitatively the population size can be predicted (as predicted active cells, pac) on the basis of the time lapse before there is recognition of one of the standard reaction patterns. Qualitatively the community can be subdivided by the signature generated from the reaction patterns observed over the period that Bart tester is incubated. This signature generated by the sequence of reaction patterns now forms the standardised bacteriological community code (SBCC). This can then be used to identify to some extent the nature of the bacterial communities in the sample and is further clarified in section 3.13.3 for the various Bart testers.

Table 3.13.1 Relationship of E-tATP values determined by sample testing on bacterial communities

Lowest E-tATP	Highest E-tATP	Bacterial Population Status	
		Active	Passive
0	20	None	N.D.
21	50	Few	
51	100	Detectable	
101	1,000	Low	
1,001	10,000	Moderate	
10,001	100,000	High	
100,001	500,000	Very High	
500,001	1,000,000	Very Aggressive	
>1,000,000		Extremely active	

Note: E-tATP ranges are given in pg/mL or g; active describes the potential metabolic activity when E-tATP are detected in that range; and ND indicates that the passive bacteria are not detectable since the populations would not be actively metabolizing and producing total ATP.

Differentiation of the potential communal activity that could occur in the sample does not relate to the acquired E-tATP levels detected and would include those passive bacteria that do become active when placed in the selective cultural environment of an appropriate Bart tester. The role in this second stage of the trigger is to determine the potential culturable bacterial activity which would be expected to be greater than the E-tATP activity since it is based on all of selectively culturable cells within the sample under examination.

In the final level in the identification of the communal structure in the sample the approach uses the rapid agitation microbiological identification inc (RASI-MIDI) protocol. Here a "fingerprint" is generated of the bacteriological community using the fatty acid methyl esters (FAME) extracted from the cell walls. This method involves gas chromatography identifying all of the C5 to C20 fatty acids and their relative abundance. MIDI has developed standard libraries for the identification of microorganisms at the genus and species level. DNI is developing RASI-MIDI based libraries for the identification of bacterial communities. Using the SLYM- based RASI the library now contains 3,892 sets of data relating to different bacterial communities (Table 3.12.2). Each community being identified is then based on the similarity to other communities retained within the same library. For comparison the standard measurement is the significance index (SI) using the FAME fingerprints in the library with that generated for the unknown. Here a perfect match would have an SI of 1.0. This is a rare event. In general, SI values of greater than 0.85 are considered very good matches and below 0.75 not significant. In the RASI-MIDI DBI library the culture medium of choice is that used for the SLYM- Bart tester (see section 3.3 for more details on the medium). Most of the evaluations has involved a four hour 8rpm in the ENH- Bart tester at room temperature followed by static incubation commonly at 30^{o}C for a sufficient period to allow recognizable turbidity to develop that would allow the MIDI identification

Table 3.12.2 Size of the DBI RASI-MIDI library for community identification

Community	Library size	Community	Library size
Wastewater (BOD)	940	**Microbial Fuel Cells**	740
Culture media	155	**Patina**	99
Bacterial cultures	104	**Salt extraction mines**	76
Bioremediation	65	**Miscellaneous***	1,713
Total Calibrations	**1,271**	**Total MIDI**	**3,892**

Note: Miscellaneous* includes RASI-MIDI identifications performed blood (human), granular activated carbon, coral, drywall (sheetrock), cooling towers, gas storage cavities, ochres, rain, snow, and cultured rusticles; in these applications the prime focus was to determine the variability (by SI) within these various bacterial communities.

3.13.3 Generation of BSCC from Bart generated data.

Each BSCC (bacteriological standardised community code) has been generated as a seven digit code (see table 3.13.2 for specific definitions and surrounding text for details). These BSCC define the dominant bacterial communities commonly found in the various habitats monitored by DBI and various Bart tester users. Each BSCC is followed by a short description of the dominant communities found that generate that particular BSCC. Nature is well known possess a unique combination of simplicity and variability. This combination means that there will always be BSCC that do not quite fit into the standardised format. To make the BSCC that much more comprehensive and "fool proof" then such experiences should be reported to DBI.

IRB ==========

IR2350C

Iron oxidizing slime forming bacteria that form carbonates in the IRB- testers.

IR2350X

Iron oxidizing slime forming bacteria that do not form carbonates in the IRB- testers.

IR2135C

Interfacial iron oxidizing bacteria dominated by slime formers and also bacteria that are able to that form carbonates oxidatively in IRB- testers.

IR2135I

Interfacial iron oxidizing bacteria dominated by slime formers and also bacteria that are not able to that form carbonates oxidatively in IRB- testers

IR2140C

Interfacial iron oxidizing bacteria dominated by dense slime formers and also bacteria that are able to that form carbonates oxidatively in IRB- testers

IR2140I

Interfacial iron oxidizing bacteria dominated by dense slime formers and also bacteria that are not able to that form carbonates oxidatively in IRB- testers

IR21430

Iron oxidizing bacteria dominated aerobically by dense slime formers and also bacteria that are not able to that form carbonates oxidatively in IRB- testers

IR2143C

Iron oxidizing bacteria dominated aerobically by dense slime formers and aerobic iron related bacteria that are able to that form carbonates oxidatively in IRB- testers

IR2146I

Interfacial iron oxidizing bacteria dominated by dense anaerobic and aerobic slime formers and also these bacteria are not able to that form carbonates oxidatively in IRB- testers

IR2146C

Interfacial iron oxidizing bacteria dominated by dense anaerobic and aerobic slime formers and also these bacteria are able to that form carbonates oxidatively in IRB- testers

IR2430C

Iron oxidising bacteria with dense anaerobic slime formers dominating over aerobic slime formers with the generation of carbonates

IR2340O

Iron oxidising bacteria with aerobic slime formers dominating over dense anaerobic slime formers but without the generation of carbonates

IR2430I

Interfacial iron oxidising bacteria with dense anaerobic slime formers dominating over aerobic slime formers but without the generation of carbonates

IR2435C

Iron oxidising bacteria with dense anaerobic slime formers becoming dominating by very aerobic slime formers with the generation of carbonates

IR2435O

Iron oxidising bacteria with dense anaerobic slime formers becoming dominating by very aerobic slime formers but without the generation of carbonates

IR2460C

Iron oxidising bacteria with dense anaerobic slime formers becoming dominating by pseudomonad bacteria with the generation of carbonates

IR2460O

Iron oxidising bacteria with dense anaerobic slime formers but becoming dominating by pseudomonad bacteria without the generation of carbonates

IR1437C

Iron reducing bacteria with dense anaerobic slime formers but becoming dominating by enteric bacteria with the generation of carbonates (coliform bacteria may be present)

IR1437R

Iron reducing bacteria with dense anaerobic slime formers but becoming dominating by enteric bacteria without the generation of carbonates (coliform bacteria may be present)

IR1465C

Iron reducing bacteria with dense anaerobic slime formers but becoming dominating by pseudomonad bacteria coupled with very aerobic slime formers generating a BR reaction with the generation of carbonates

IR1465I

Iron reducing bacteria with dense anaerobic slime formers but becoming dominating by pseudomonad bacteria coupled with very aerobic slime formers generating a BR reaction with the generation of carbonates

IR1400C

Iron reducing bacteria with dense anaerobic slime formers with the formation of carbonates

IR1400R

Iron reducing bacteria with dense anaerobic slime formers without the formation of carbonates

IR1460C

Iron reducing bacteria with dense anaerobic slime formers and a secondary dominance of pseudomonads with the formation of carbonates

IR1460R

Iron reducing bacteria with dense anaerobic slime formers and a secondary dominance of pseudomonads without the formation of carbonates

IR1470C

Iron reducing bacteria with dense anaerobic slime formers and a secondary dominance of enteric bacteria with the formation of carbonates (coliform bacteria may be present)

IR1470R

Iron reducing bacteria with dense anaerobic slime formers and a secondary dominance of enteric bacteria without the formation of carbonates (coliform bacteria may be present)

IR1350C

Iron reducing bacteria with dense slime formers and a secondary dominance of very aerobic slime forming bacteria with the formation of carbonates

IR1350I

Iron reducing bacteria with dense slime formers and a secondary dominance of very aerobic slime forming bacteria without the formation of carbonates

IR1230C

Iron reducing bacteria with a secondary dominance of slime forming iron oxidising bacteria with the formation of carbonates

IR1230I

Iron reducing bacteria with a secondary dominance of slime forming iron oxidising bacteria without the formation of carbonates

IR1700C

Iron reducing bacteria with a dominance of enteric bacteria with the formation of carbonates (coliform bacteria may be present)

IR1700R

Iron reducing bacteria with a dominance of enteric bacteria without the formation of carbonates (coliform bacteria may be present)

IR1270I

Iron reducing bacteria with a dominance of slime forming bacteria including enteric bacteria including some iron oxidising bacteria without the formation of carbonates (coliform bacteria may also be present)

IR1240I

Iron reducing bacteria with a dominance of slime forming bacteria including some iron oxidising bacteria without the formation of carbonates

IR1230I

Iron reducing bacteria with a dominance of dense gel-like growths but including some iron oxidising bacteria without the formation of carbonates

IR1235I

Iron reducing bacteria with a dominance of aerobic iron oxidising bacteria forming very aerobic slime growths throughout the IRB- tester

IR1430R

Transitional iron related bacteria dominated by anaerobic slime forming slime formers with some aerobic activity occurring.

SRB ==========

SR1000I

Proteolytic sulfide producers dominate with by degradation of the sulfur amino acids present within the total organic carbon. Commonly dominates at the ORP transitional zone where there is sufficient protein in the organics.

SR1300I

Proteolytic sulfide producers aggressively dominate with by degradation of the sulfur amino acids present within the total organic carbon and can cause hydrogen sulfide to be generated throughout the tester. Sulfate reducing bacteria may also be active. Commonly dominates at the ORP transitional zone where there is sufficient protein in the organics

SR2000R

Sulfate reducing bacteria dominate the sulfide producers under reductive conditions and sulfates are significantly present in the sampled environment

SR2300I

Sulfate reducing bacteria do dominate but there are some active proteolytic sulfide producers also present degrading the sulfur amino acids in the organic carbon present in the sample

SLYM ==========

SL1000X

Slime forming aerobic bacterial community

SL3100X

Initial slime forming bacterial activity occurs at the ORP interface with the formation of dense lateral slime plates but these rapidly disperse into aerobic slime formers that generate a cloudy reaction in the tester.

SL2100I

There is initially a dense gel-like growth in the base of the tester but this is followed rapidly by a generalised cloudy reaction throughout the tester

SL1400X

Aerobic slime forming bacteria as a cloudy reaction but then there is intense growth around the ball to form an aerobic slime ring

SL5140X

Initially there is aerobic thread-like growths that form vertically between the floating ball and the base of the tester. These threads generally disrupt to form cloudy growths but with the co-development of an aerobic slime ring. This bacterial community therefore includes an initial dominance of thread formers that can last commonly for one to two days followed by dispersed growth and the development of the aerobic biomass cap ringing the ball.

SL1600X

Aerobic slime formers are generated throughout the liquid sample to form clouding. This is followed by a pale blue UV fluorescence that commonly occurs from the equator of the ball downwards by 15 to 25mm and usually lasts 2 to 5 days. This is indicative of the presence of *Pseudomonas aeruginosa*

SL1700X

Aerobic slime formers are generated throughout the liquid sample to form clouding. This is followed by a pale blue UV fluorescence that commonly occurs from the equator of the ball downwards by 20 to 35mm and usually lasts 3 to 10 days. This is indicative of the presence of *Pseudomonas fluorescens*

SL1800R

Slime forming bacteria that are primarily functioning fermentatively (reductively) with the generation of gases which collect as a foam ring around the ball

SL1000B

Anaerobic slime forming bacteria that gradually shift the environment in the tester to become very reductive with the reduction of organics to carbon-rich daughter products such as petroleum hydrocarbons and elemental carbon

SL4510X

Aerobic slime forming bacteria that initially from a very aerobic biomass as a slime ring around the ball followed by thread-like growths down through the culturing liquids with the final generation of a cloudy growth throughout the cultured liquid in the tester

SL2000I

Anaerobic dense slime forming bacteria do not adapt to aerobic conditions and remain as an entrenched viscid gel growth in the lower part of the culturing liquids in the tester.

SL2000B

Anaerobic dense slime forming bacteria do not adapt to aerobic conditions and remain as an entrenched viscid gel growth in the lower part of the culturing liquids in the tester. This gradually shifts the environment in the tester to become very reductive with the reduction of organics to carbon-rich daughter products such as petroleum hydrocarbons and elemental carbon

SL2100B

Anaerobic dense slime forming bacteria grow but then adapt to aerobic conditions by clouding the culturing liquids in the tester. This intense activity now gradually shifts the environment in the tester to become very reductive with the reduction of organics to carbon-rich daughter products such as petroleum hydrocarbons and elemental carbon

HAB ==========

HA1000X

Aerobic heterotrophic (“organic busters”) bacteria dominate which degrade organics with the main product being carbon dioxide. Oxidative respiratory mechanisms cause the complete degradation of organics.

HA1000B

Aerobic heterotrophic ("organic busters") bacteria initially dominate to degrade organics with the main product being carbon dioxide. However anaerobic fermentative then ensues with the reduction of organics to carbon-rich daughter products such as petroleum hydrocarbons and elemental carbon

HA2000R

Anaerobic heterotrophic ("organic busters") bacteria initially dominate to degrade organics with the main products including fatty acids. Reactions of this type (DO) are initially unstable and the DO reaction should only be declared when there is a clearly descending reduced (bleached) zone moving from below the ball downwards.

HA2000B

Anaerobic heterotrophic ("organic busters") bacteria initially dominate to degrade organics with the main products including fatty acids. Reactions of this type (DO) are initially unstable and the DO reaction should only be declared when there is a clearly descending reduced (bleached) zone moving from below the ball downwards. The environment in the tester becomes very reductive with the reduction of organics to carbon-rich daughter products such as petroleum hydrocarbons and elemental carbon

AP ==========

AP1000R

Anaerobic fermentative bacteria generate fatty acids as the principal daughter products which will then lower the pH into the mildly acidic range of 4.5 to 5.8

AP1200I

Anaerobic fermentative bacteria generate fatty acids as the principal daughter products which will then lower the pH into the mildly acidic range of 4.5 to 5.8 but these then become degraded with the pH returning into the neutral range

DN ==========

DN 1000R

Anaerobic fermentative bacteria that are capable of reducing nitrates and nitrites (denitrifiers) to more reductive forms with the eventual generation of nitrogen gas that becomes entrapped around the ball to form a foam ring which commonly lasts one to three days.

N ==========

NO1000X

Nitrosofiers (*Nitrosomonas*) are present in the aerobic community but are not dominant

NO2000X

Nitrosofiers (*Nitrosomonas*) are very active as an active part of the aerobic community

NO3000X

Nitrosofiers (*Nitrosomonas*) are dominating in the aerobic bacterial community and are able to generate excessive levels of nitrite and nitrate that become resident in the sample's environment.

LG ==========

LG1000X

Micro-algal community that generates a grass green color and predominantly grows on the surface of the sample and also at contact points with solid surfaces

LG1260I

Algae mature over time from rom a grass green to a fuzzy growth and at the samples edge with gradual darkening of the green as the cyanobacteria dominate

LG2000X

Here the growth forms as a consistently fuzzy green growth

LG2100X

Fuzzy green growths shift over time to grass green with a cleaner differentiation of the borders of the growths which does not darken

LG2600X

Fuzzy green growths shift to dark green forms which has more defined edges

LG3000X

Growths appear as predominantly hues of orange to brown without the development of green chlorophyllous forms.

LG4000X

Yellow to brown colors dominate the growths which do not mature to shades of green

LG5000X

Grass green flocculent growths of algae form predominantly on the floor of the tester

LG5600I

Grass green flocculent growths of algae form predominantly on the floor of the tester that gradually shifts to dark green colors as the cyanobacteria become dominant

LG6000I

Growths are dark green in form and gradually grow over time. These are dominated by the Cyanobacteria

PL ==========

PL1000X

Slime forming aerobic bacterial community that dominates the tester rapidly

FL ==========

FL1000X

Slime forming aerobic bacterial community that dominates the tester rapidly

FL1200X

Aerobic slime formers are generated throughout the liquid sample to form clouding. This is followed by a pale blue UV fluorescence that commonly occurs from the equator of the ball downwards by 15 to 25mm and usually lasts 2 to 5 days. This is indicative of the presence of *Pseudomonas aeruginosa*

FL1300X

Aerobic slime formers are generated throughout the liquid sample to form clouding. This is followed by a pale blue UV fluorescence that commonly occurs from the equator of the ball downwards by 20 to 35mm and usually lasts 3 to 10 days. This is indicative of the presence of *Pseudomonas fluorescens*

3.14 Pantone Color Recognition for the Bart Tester Reactions

3.14.1 Introduction

This section addresses the use of the Pantone RGB colors to defined the various Bart reactions

Table 3.14.1 Pantone recognition for the IRB- test

Bart	Reaction	Pantone	R	G	B
IRB	**WB**	7541	89	89	90
	DG	360	35	74	39
	DG	362	29	56	30
	Y	7403	94	82	51
	Y	7405	87	68	2
	FO				
	CL	7403	94	82	51
	CL	7405	87	68	2
	BC	729	75	56	45
	BC	730	65	48	37
	BG	463	52	44	32
	BG	4635	56	42	34
	BG	464	58	48	33
	BG	4645	63	47	39
	BG	576	43	56	35
	BG	7497	54	51	44
	BR	7516	61	42	33
	BR	7517	53	36	27
	GC	360	35	74	39
	GC	364	33	47	30
	RC	485	86	34	27
	RC	484	60	34	29
	BL	7532	43	38	33

Table 3.14.2 Pantone recognition for the SRB- test

Bart	Reaction	Pantone	R	G	B	color
SRB	**BT**	BLACK 6	32	32	35	
	BB	BLACK 6	32	32	35	
	XR1	5315	86	82	35	white
	XR2	5305	79	75	82	grey
	XR3	5315	86	82	87	grey
	XR4	5295	70	66	55	grey
	XR5	5015	79	61	62	beige

Table 3.13.3 Pantone recognition for the SLYM- test

Bart	Reaction	Pantone	R	G	B	color
SLYM	**DS**	7537	71	71	67	base only
	CL	7537	71	71	67	
	BL	7532	43	38	33	
	TH	7539	63	62	60	limited structures
	PB	7453	58	65	86	
	GY	367	55	83	41	

Table 3.14.4 Pantone recognition for the HAB- test

Bart	Reaction	Pantone	R	G	B	color
HAB	**Control**	3145	0	47	53	control A
	Control	312	0	67	83	control B
	(+) 1	7404	8	89	69	yellow
	(+) 2	7402	95	88	63	yellow
	(+) 3	7403	94	82	51	yellow
	(+) 4	7404	96	83	27	yellow
	(+) 5	5315	86	82	35	white
	(+) 6	5315	86	82	35	grey

Note: Control A or B are the blue colors seen in a negative reaction, (+) show the colors that are commonly generated when a positive reductive process has occurred.

Table 3.14.5 Pantone recognition for the APB- test

Bart	Reaction	Pantone	R	G	B	color
APB	**Control:**	513	62	31	56	purple
	(+) 1	127	96	87	45	yellow
	(+) 2	128	96	82	35	yellow
	(+) 3	129	95	70	92	yellow

Table 3.14.6 Pantone recognition for the N- test

Bart	Reaction	Pantone	R	G	B	colour
N	PP (ball)	514	80	48	73	pink
	PP (ball)	515	88	58	80	pink
	PP (sol)	705	96	83	27	pink
	RP (ball)	7425	77	36	45	lt. Red
	RP (sol)	706	100	79	82	lt. red
	DR (ball)	7426	72	31	29	red
	DR (sol)	708	98	55	60	red

Table 3.14.7 Pantone recognition for the ALGE- test

Bart	Reaction	Pantone	R	G	B	color
ALGE	GG	555	34	48	42	green
	FG	555	34	48	42	green patches
	OB	7425	77	36	45	orange-brown
	OB	715	97	59	36	orange-brown
	OB	7525	60	46	40	orange-brown
	YB	7404	96	83	27	yellow brown
	YB	728	82	64	51	yellow brown
	GF	554	32	42	38	green floc
	GF	555	34	48	42	green floc
	DG	582	53	55	25	dense green
	DG	567	31	40	37	dense green
	DG	5535	28	33	31	dense green

Table 3.14.8 Pantone recognition for the FLOR- test

Bart	Reaction	Pantone	R	G	B	color
FLOR	**GY**	367	55	83	41	Green yellow
	PB	7453	58	65	86	Pale blue

Table 3.14.9 Pantone recognition for the POOL- test

Bart	Reaction	Pantone	R	G	B	color
POOL	CL	5793	76	77	65	grey

3.14 Bart predicted active cells population summary

This section forms a summary of the relationship between time lapse and the predicted active bacterial population (as pac/mL) which are taken to be equivalent to colony forming units per mL, cfu/mL) Each tester type indicates the color of the cap and then the predicted population considered significant for that particular bacterial community.

Table 3.14.10 RED CAP IRB – Tester for Iron Related Bacteria

Daily Observations of the IRB-BART tester showing variability in Predicted Populations (pac/mL)

Days:	**1**	**2**	**3**	**4**
High population	1,990,000,000	494,000	122,000	30,000
Average population	**598,000,000**	**140,000**	**34,800**	**8,630**
Low population	161,000,000	40,000	9,900	2,450

Days:	**5**	**6**	**7**	**8**
High population	7,500	1,850	460	114
Average population	**2,100**	**529**	**131**	**32**
Low population	608	150	37	9

Table 3.14.11 BLACK CAP SRB – Tester for Sulfate Reducing Bacteria

Daily Observations of the SRB- BART tester showing variability in Predicted Populations (pac/mL)

Days:	**1**	**2**	**3**	**4**
High population	8,180,000	1,800,000	434,000	99,900
Average population	**2,180,000**	**502,000**	**115,000**	**26,600**
Low population	283,000	134,000	30,800	7,100

Days:	**5**	**6**	**7**	**8**
High population	23,000	5,290	1,220	280
Average population	**6,130**	**1,410**	**325**	**74**
Low population	1,630	376	96	19

Table 3.14.12 LIME GREEN SLYM – Tester for Slime Forming Bacteria

Daily Observations of the SLYM- BART tester showing variability in Predicted Populations (pac/mL)

Days:	1	2	3	4
High population	7,510,000,000	1,470,000	288,000	56,500
Average population	**1,730,000,000**	**339,000**	**66,500**	**13,000**
Low population	399,000	78,300	1,630	3,000

Days:	5	6	7	8
High population	11,000	2,160	424	83
Average population	2,550	500	97	19
Low population	588	115	22	4

Table 3.14.13 OXFORD BLUE CAP HAB – Tester for Heterotrophically Active Bacteria (22°C)

Daily Observations of the HAB- tester showing variability in Predicted Populations (pac/mL) using 22±2 °C

Days:	1	2	3	4
High population	40,600,000	4,310,000	458,000	48,700
Average population	**5,390,000**	**573,000**	**60,900**	**6,470**
Low population	717,000	76,200	8,100	860

Days:	5	6	7	8
High population	11,000	2,160	424	83
Average population	**2,550**	**500**	**97**	**19**
Low population	588	115	22	4

Table 3.14.14 OXFORD BLUE CAP WW - HAB – Tester for Heterotrophically Active Bacteria (22°C) for Sanitary Wastewaters with Selected Hourly Observations of the WW-HAB- tester showing variability in Predicted Populations (pac/mL) at 22±1 °C with observational readings in hours.

Hours:	4	8	16	24
High population	83,100,000,000	15,830,000	3,010,000	109,000
Average population	**23,900,000**	**4,570,000**	**165,000**	**6,020**
Low population	6,910,000	251,000	9,120	37

Hours:	32	40	48	64
High population	724,000	16,000	253	4
Average population	**22,000**	**416**	**7**	**1**
Low population	1,000	12	1	1

Table 3.14.15 OXFORD BLUE CAP E - HAB – Tester for Heterotrophically Active Bacteria (28°C) In the Environment with Selected Hourly Observations of the E-HAB- tester showing variability in Predicted Populations (pac/mL) using 28±1 °C with observational readings in hours.

Day:	1	2	3	4
High population	100,000,000	5,700,000	250,000	38,000
Average population	**9,000,000**	**320,000**	**45,000**	**11,020**
Low population	408,000	53,000	13,000	4,000

Days:	5	6	7	8
High population	10,000	3,500	1,500	710
Average population	**4,000**	**1,500**	**750**	**400**
Low population	2,000	800	400	240

Table 3.14.16 PURPLE CAP APB – Tester for Acid Producing Bacteria (22°C)

Relationship between time lapses (days) and predicted population (pac/mL) of APB

Day:	1	2	3	4
High population	2,290,000	397,000	68,900	11,900
Average population	**473,000**	**82,100**	**14,200**	**2,470**
Low population	97,800	16,900	2,940	510

Days:	5	6	7	8
High population	2,070	359	62	10
Average population	**428**	**74**	**12**	**2**
Low population	88	15	2	1

3.14.17 GREY CAP DN– Tester for Denitrifying Bacteria (22°C)

Relationship between time lapses (days) and predicted population (pac/mL) of DN.

Day:	1	2	3	4
High population	2,290,000	397,000	68,900	11,900
Average population	**473,000**	**82,100**	**14,200**	**2,470**
Low population	97,800	16,900	2,940	510

Days:	5	6	7	8
High population	2,070	359	62	10
Average population	**428**	**74**	**12**	**2**
Low population	88	15	2	1

Table 3.14.18 DARK GREEN CAP ALGE – Tester for MICRO – ALGAE

(22°C)

Relationship between time lapse (days) and predicted population (pac/mL) of micro-algae using the ALGE- Tester

Days:	1	4	8	12
High population	315,000	101,000	22,400	4,970
Average population	**224,000**	**72,400**	**16,000**	**3,540**
Low population	159,000	51,500	11,400	2,540

Days:	16	24	32
High population	1,100	53	2
Average population	**783**	**38**	**1**
Low population	558	27	1