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Standard Methods for the Application of BART testers in the Environmental Investigations of Microbiological Activities.

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Background

This document forms a compendium of the "*Standard Methods for the Application of BART testers in Environmental Investigations of Microbiological Activities*". BART testers have been used commercially for over two decades by consultants, engineers and plant operators who wish to determine the nature of bacteriological activity that is affecting their results. While traditional microbiology laboratories can attempt some of these analyses the BART has the advantage of being user friendly and generates results that are often directly applicable to the perceived challenge.

The BART tester comes in two basic forms for use in the field (field tester) and laboratory (lab tester). Here the field tester is more durable and hence more expensive while the lab tester is "bare bones" for use in a laboratory setting with trained microbiology technicians. Both testers share the same basic premise: (1) examine for the selected activities of the bacterial communities in the sample; (2) set up inside the tester different environments from reductive nutrients rich in the base to oxidative-nutrient poor in the top of the sample in the tester; (3) use the sample undiluted as 15ml if liquid, and if solid then use between 0.15 and 1.5g of material with make up to 15ml (total) using a recommended diluant; (4) under no circumstances should the tester be agitated or shaken except where included as a part of the protocol (see HAB- and APB- for examples of this); (5) all incubation (growing of the bacteria in the tester) is performed at room temperature ($22\pm2^{o}C$) as a matter of convenience even though no bacteria commonly grow fastest at this temperature; (6) different BART testers are designed to specifically detect different communities of

bacteria (called consorms); and (7) all testers are sterile in their manufacture and are subject to ISO 9001:2000 certification.

For the bacterial testing the BART is primarily used to determine whether the sample possesses activities that relate to the selected bacterial consorm. Here the activity is determined by the time lapse (length of time) that occurs before a reaction is recognized and recorded for interpretation. In the act of interpreting the time lapse into predicted active cells (or colony forming units) then different programmes can be used which utilize the same final formula. These programmes include "QuickPop" (functions only at 22±2°C and allow time lapses in seconds, hours or days to be converted to populations; BART-SOFT (version 6) which allows all of the data to be incorporated into an archived form which includes prediction of the population based on the first recognized reaction occurring; and Visual BART reader (VBR) that is a fully automatic system employing a camera and computer and allows the operator to review all reactions and activities that have occurred in the testers for interpretation including population prediction.

In the analytical use of the BART (biological activity reaction tester) the prime focus needs to be the recognition of the first activity or reaction that is recognized in the "Certificate of Analysis" for that BART type. While subsequent reactions and activities are recognized only the first phase reactions are employed. For the IRB- BART tester the first reactions (phase 1) are not used since these relate to physico-chemical reactions between the tester and the sample and there are three further phases of reactions (phase 2, phase 3, and phase four) reactions which are used in the generation of a reaction pattern signature and prediction of the population (from the generated time lapse).

This document is separated into: (1) primary and secondary objectives for using the BART testers; (2) physical characteristics significant to the functioning of the BART tester; (3) descriptions of the individual BART testers that are in significant production in 2010; (4) significant factors affecting the BART testing practise; (5) preparation of samples for BART testing; (6) disposal of completed BART testers; (7) specialty uses for the BART testers; (8) how the BART tester functions; (9) basis BART parameter codes (p codes) used to describe each of the testers; (10) BART parameter codes; (11) conversion tables to determine risk; and (12) innovative application of the biotesters in various environments.

Chapter 1

Introduction

This document forms a summarized protocol for the use of all BART™ testers presently in commercial production by Droycon Bioconcepts Inc. This protocol therefore supersedes all previous methods and documentation appearing in print or on the web site, www.dbi.ca. Previous documentation remain valid descriptions of the testers and the methods recommended for analysis but this document represents a more comprehensive description of the methodologies and the findings leading to the production of data and findings.

1.1. Primary objective:

All new protocols use room temperature (22±2°C) for the incubation/ growth/ culture of the bacteria in the sample that are targeted by the environmental conditions presented in the BART tester. It is recognized that the choice of this temperature is based on convenience since this is the normal room temperature range in offices, workshops and laboratories. It is recognized in setting that temperature as one of convenience it is not an ideal temperature for maximising the growth of bacteria (Cullimore, 2008, appendix J).

1.2. Secondary objectives:

1.2.1 It is possible to grow BART testers at other temperatures and the only other temperature recognized is 28±1°C which is close to optimal for many bacteria in the temperate environments. To

perform these tests then an incubator has to be employed or a small heated room that temperature that can be controlled by an electronically controlled recirculating heater.

1.2.2. BART testing methodologies can employ the video BART reader system (VBR). This analytical system utilises a camera (for periodic digitization of images), BART rack that holds the laboratory version testers (30, or 60), and a small computer that carries the VBR software and allows interpretation. The imaging system is applicable to the following BART tester types: IRB-, SRB-, SLYM-, HAB-, DN-, APB-, and ALGE- (it should be noted that the ALGE- BART tester requires an additional 24days). The N- BART tester is not suitable for the VBR system since there is a fixed time lapse of five days followed by colorimetric chemical analysis for the presence of nitrite (presence indicate nitrifying bacteria are present).

1.2.3. The recommended incubation temperature for convenient testing is room temperature (22±2°C). It should be noted that both the QuickPop and VBR systems allow the statistical prediction of the population size based solely on the first generated time lapse observed in the tester. This time lapse is triggered by the observation of a specific reaction pattern in the tester

1.2.4. Bacterial populations are presented as predicted active cells per ml (pac/ml) in each ml of the fifteen ml employed in the sample. These predictions are calculated using specific equations generated for each of the BART tester types to relate time lapses to the recognizable levels of specific bacterial activities. For each BART tester type the formulae are generated by a combination of suitable cultural test methods commonly involving bacterial communities (consorms) and selected mixed or pure cultures of

bacteria. Most test methods employ the use of selective agar spreadplate technologies which generate data as colony forming units (cfu). For the equations (see 1.2.3. above) these data (cfu and time lapse) are used to create the pac which may be considered equivalent to cfu. Election of the pac as a common term is based on the premise that the BART tester only reacts to the presence of bacterial cells that are active in the sample.

1.2.5. Bacterial communities (consorms) are recognized by the occurrence of bacterial activities within the tester using the selective standard medium deposited into the base of the BART tester. Reactions are recognized as being limited only to those standard reactions described in the "Certificate of Analysis" document that accompanies every box of BART testers. Activities are determined by the time lapse to the first recognized reaction achieved by visual daily observation (semi-quantitative) or using the VBR system in which case the common time interval between digital recording of the image is 15minutes. This frequency of recording allows quantitative of the population (when observed) expressed in pac.

1.2.6. It has to be recognized that every sample subjected to BART analysis involves the generation of some unique color and clarity shift. Whether using visual observation or the digital VBR system the colors rendered as relating to a given activity are subject to variation. Such variations are expressions of the reactions generated by the particular consorm(s) within the sample. For this reason the expressed colors or clarity shift in a recognized reaction are only given in the form of two letters that best represents the observers opinion of the reaction. If the reaction has been determined by the VBR system then that particular image can be converted into a .jpg image

and sent to DBI for further confirmation of the designated reaction type.

1.2.7. Storage of the testers are routinely in sealed foil pouches which have tear down tabs for breaking open the pouch. While sealed in the aluminum foil pouch there is extremely limited ability water to enter. These pouches are presented in threes for the field testers but each tester is preserved in a separate pouch. For the laboratory tests they are sealed as five testers in a common pouch and once opened then all five lab testers should be used as quickly as possible with those not being used being returned to the foil pouch which is rolled up and sealed using adhesive tape and then stored in a cool dry refrigerator for use after no longer than three weeks. Care should be taken when handling the foil pouches that they are not punctured in any manner since this would allow moisture to enter the tester. Compromise can also be caused by ripping the tear down too violently in one of the three field testers joined in a common set of pouches. Here the ripping could tear the foil of a neighbouring pouch and allow moisture to enter. Under no circumstances should the pouches be cut using scissors or casually ripped open since this would increase the probability of puncturing neighbouring sealed pouches. Do not attempt to bag the unused testers in plastic bags or sheets since these do not effectively protect the contents of the tester from moisture. If moisture has entered a BART tester then the selective nutrient pellet in the base of the tester can swell and become mobile. This is one of the reasons for keeping the testers vertical during storage. If the testers are laid horizontally then the moisture will cause the media to move up the inside walls of the tester and stick to the BART ball.

Chapter 2

Characteristics of the BART tester

2.1 Physical Characteristics

The prime feature of the BART tester is that it utilizes a 15±0.2ml whole liquid or diluted sample. One unique feature of the tester is a floatable BART ball that has a diameter of 19.7±0.1mm and a density of 1.08±0.01 and hence floats in non-saline water with 80±2% of the ball submerged. For the inner tube of the BART tester the inner walls are sloped slightly outwards and at the floatation point for the charged tube where the BART ball would float then the inside diameter is 22.1±0.1mm meaning that there is a gap between the floating ball and the wall of the inner tube that averages 1.2±0.02mm when the ball is centrally positioned. It is this floating ball that restricts the movement headspace gases (e.g. oxygen) down into the charged water sample.

There are two zones created by the floating BART ball: (1) liquid above the equator of the ball and below the exposed surface of the ball; and (2) liquid below the equator of the ball. Volume above the equator (1) and directly exposed to the headspace gas is 0.33±0.02ml, has a surface area of 2.21±0.2cms^2, and an aspect ratio (surface area: volume) of 1: 0.14 indicating that conditions would be very oxidative. Volume below the ball equator was 14.8±0.2ml with the

"surface area" at the equator interposed with the oxidative zone above was 0.788±0.02cms^2 which gave an aspect ratio of 1: 18.4±0.2 which would generate reductive conditions since headspace oxygen would have to diffuse past the throat of the ball (i.e. at the equator of the ball which would be the closest point to the vertically sloped walls of the tube. In summary the charged BART tester has two zones of sample divided by the equator of the BART ball. 2.2% of the volume (0.33ml) of the liquid sample is set within an oxidative zone above the equator and the headspace atmosphere. The remaining liquid sample (97.8% or 14.67ml)) is below the BART ball equator and so is more likely to become reductive as any oxygen present in the water is used for respiratory functions by the intrinsic flora. Metabolism then moves to a reductive type involving fermentation. These reductive conditions are likely to first form in the base of the BART tester where the selective chemical nutrient pellet is dissolving and diffusing upwards into the water column. These events mean that the oxidative-reductive potential (ORP) interface slowly moves up the tester as the oxygen is utilized and often with intense bacterial activity around the interface.

One unique quality of the BART tester is that it presents to the bacteria in the tester a unique and dynamic series of environments as the ORP interface moves up flows the utilization of the oxygen in the tester and creates increasingly reductive conditions occurring underneath. At the same time the selective chemical nutrient pellet is dissolving and diffusing upwards into the tester's water column. This event may trigger the focussed bacterial accumulation and degradation of the chemicals with the creation of a biomass that may be observed firstly as a gel-like deposit, plate-like mobile growth or the generation of reductive conditions (HAB- BART tester) or acidic conditions (APB- BART tester). The speed with which these activities are observed can be directly measured as the time

lapse and the type of bacteria involved determined by the reactions observed. It should be remembered that BART stands for bacteriological activity (generated by the time lapse) and reactions (generated by the recording of specific reactions within the tester).

Each BART tester product type is decided by the type of dried chemical nutrient pellet incorporated into the base of the tester and they may be used with water based liquid samples (15ml) or semi-solid, porous, solid, or emulsified samples. In these latter cases then dilution or dispersion in a sterile water base is necessary to reach the 15ml of liquid volume needed for the BART tester to function correctly.

All BART testers incorporate the inner tube which is balled, incorporates the selective culture medium in the base of the tube as a sterile dried crystalline deposit, and then capped. The weight of the inner BART tester (laboratory version) without the medium is 16.03±0.03g (without labelling). When the field version of the BART tester is prepared then this includes an inner tester which is identical to the laboratory version but includes a second outer tube and cap to allow effective use of the tester under the more rigorous conditions of the field. These field units weight 36.12±0.03g without labelling and media.

To protect the BART testers (field or laboratory versions) from humidity causing the dehydrated media pellets to absorb water, expand and possibly become mobile, all testers are packed and stored in sealed aluminum pouches. Here the foil used was 5±0.2mil in thickness and was sealed using a heat sealer set at 300±10°C with a three second compression time. Pouches sealed in this manner were found to be moisture proof. Two pouches routinely used are the triple pouch for the field testers (190 x 300mm, 7.3 x 11.5”) and the single pouch which holds five laboratory testers (220 x 160mm, 8.5 x 6.15”). Weight of aluminum

foil used per laboratory tester is 1.760±0.005g and additional aluminum is employed when packaging each of the field testers (5.031±0.01g).

Boxes of either the laboratory or field versions are shipped in a common cardboard box that includes either fifteen (15) laboratory testers in three aluminum bags of five testers, or three of the triple pouches which then contained a total of nine (9) field testers all sealed separately. These boxes accommodate either testers and each box includes a "Certificate of Analysis" that includes the batch serial number that is generated through the ISO 9001:2000 certified procedures for every particular batch of 650 BART testers. Cardboard boxes for the testers are 117 x 143 x 286mm (4.5" x 5.5" x 11"). Some distributers label the outside of the box with the expiration date but all "Certificates of Analysis" includes the expiration date. Boxes should be kept in a cool dry environment until used. The shelf life has an expiry data set four years after the packaging of the testers into the sealed foil pouches. Quality management of the packaging through to boxing and warehouse storage follows the manufacture's procedures. Testers are manufactured under sterile clean room conditions and all subsequent procedures are monitored to ensure that the final product sealed into the aluminum pouch remains internally sterile.

There are therefore two basic presentations of the BART tester in boxed sets of nine testers for use in the field; and boxed sets of fifteen for use in the laboratory. These are known respectively as "field" and "laboratory" testers. Field testers are designed to provide additional protection to the inner BART tester under the more rigorous conditions of field use.

Chapter 3

BART Tester Definitions

One of the principal factors affecting the success of the BART testers over the last twenty years has been the relatively simple ability to detect selected bacterial activity. In large part this is based upon 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 (consorms) that are the subject of that specific investigation. This Chapter addresses the form of the proprietary chemical nutrient pellet and the weight incorporated into a single inner BART tester. Included is the definition of the proprietary formula involved, the weight when dried following the standard ISO 9001: 2000 protocols, and a brief definition of the of the bacterial types that can be detected using that specific BART tester. All media are dried to a constant weight at $52 \pm 2^{o}C$ with a drying time standardized at eight days. Each BART tester type employs one (laboratory version) or two (field version) caps that are colored in a distinctive manner to separate the BART tester types.

3.1.1. 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). Some bacteria can perform both functions and so the definition of iron related bacteria is broad (Cullimore, 2010):

> "Incorporates all bacteria that are able to accumulate 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 generated over the diffusing basal pellet and if these bacteria dominate then the whole column may appear 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 will have an orange tinge. In both cases the sample column remains relatively clear (free from clouded growths) and this is not used the prime differentiation in this tester (see also 3.1.2.1.).

IRB- BART pellet dried weight per tester: 349.5±1.5mg

Colors where relevant to the interpretation of any given BART reaction are described incorporating the Xerox Pantone® colors defined in 2007 printed on July 25th.

3.1.2. 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-BART tester. These four phases make the determination of specific bacterial much more challenging than the 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 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 trigger the determination of the time lapse (and hence the predicted active cell population for that sample). Phase three now recognizes the reactions that occur in sequence during the incubation of the tester after the phase two population determinations. Phase four is a terminating event (black liquid, BL) after which no further reactions are determined and the test is considered finished.

3.1.2.1 Phase One IRB Reactions

Phase one reactions are not used in the identification of IRB bacteria as such but should be recorded and do provide some insight into the types of environmental conditions that are 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-BART testers forming between 30minutes and 10hours into the incubation period. This reaction is easy to recognize because the conical base of the tester turns white with a crystalline deposit. Time lapse photography does not reveal the deposit building up as layers but

as an event that occurs quite quickly (less than one hour). Analysis reveals that the dominant chemicals are carbonaceous. Volume occupied in the basal cone ranges around 1.2±0.2ml and once formed 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 the biofouling biomass should include acidic treatment in at least one phase to dissolve the carbonates that are likely present in 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 form of iron dominating. This would indicate that iron reducing bacteria are likely to be both present and active reducing the ferric-iron in the selective chemical nutrient pellet to ferrous. While GD can be used as evidence of ferric-iron reducing bacterial activity it is not to be used as a confirmatory reaction.
- 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 insoluble forms of ferric-iron are being mobilized as yellow colloidal material in the 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 in the development of a treatment strategy but is not diagnostic of specific IRB activities. GD

and YD reactions can be used to consider that iron-reducing or iron-oxidizing activities are occurring but they are of limited scope and require phase two confirmation.

3.1.2.2. Phase Two IRB Reactions

There are two reactions that occur in phase two and these define the type of IRB activity that is occurring in the sample. While phase one gave some implicit information, phase two generates the first recognized reaction and is then used to predict the population (as pac/ml). 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 one reactions are described:

- Foam formation (FO) is recognized only when there is a ring of bubbles (or more) forming around the BART ball. These bubbles each contain gas which is restrained within biofilms. Common gases associated with foam formations are some combination of carbon dioxide, methane, nitrogen, and hydrogen. The manner in which the foam is generated and remains is a reflection of the dominant gas within the foam. Nitrogen tends to be the most durable foam gas lasting for several days and methane is the least durable since it is rapidly degraded under the oxidative conditions that would exist in 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 moved up to that of the room. FO indicates that fermentative activities are dominating in the tester with the evolution of gas that then become wrapped in biofilms to form the bubbles that then lock into the foam layers around the BART 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 BART 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.

- Cloudiness (CL) tends to be the first reaction occurring in the IRB-BART tester where the sample has come from an oxidative environment and respiratory types of activity are occurring. Cloudiness is recognized by the loss 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 of the tester. CL is most easily recognized using the VBR tester rack which has a black line behind the testers that does show clouding as a greying of the black. In the absence of a rack then success can be achieved using black plastic disposable pen which also shows the clouding clearly against the horizontal body of the pen. 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 3.1.2.3. below)

3.1.2.3. Phase Three IRB Reactions

Phase three incorporates the bulk of the recognized reactions seen in an IRB- BART tester except FO and CL (3.1.2.2.) and BL (3.1.2.4) along with the phase one reactions (3.1.2.1.). These phase three reactions can be grouped around whether they occur reductively or oxidatively 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 but can be superceded by the phase four BL.

- Basal Gel (BG) occurs commonly in the lowest third of the IRB- BART 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 within the solution above. Commonly in a BG reaction the solution above the BG is 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 BART 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 frequently the very aerobic iron related bacteria can be microscopically observed here. BR is particularly a favourable environment for the sheathed and ribbon-forming iron oxidizing bacteria.

- 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.

3.1.2.4. Phase Four IRB Reactions

Phase four reaction is terminal and can be recognized by the sample medium column in the tester becoming a Black Liquid (BL). Closer examination of the BL reaction reveals that the blackening is caused by jet black 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).

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: ***, highly probable occurrence; **, likely to occur with a moderate probability; *, observed on some occasions but relatively uncommon; no asterisk means that this reaction does not normally occur. Sequencing of the reactions would be from phases 1 to 2 with the order of the reactions observed being of possible diagnostic significance. Consorms 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 consorms can incorporate IRB.

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.

Table 3.1.3 Probable IRB-BART tester reactions for selected bacterial consorms

	Phase 2				Phase 3			4
Consorm	**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 - 19-21 PLG	***	*	***	**	***		**	*
2 - 14-15 ROT	*	***	*			**	**	**
2 - 21-22 SLM	***		**	**	**	**		
3 - 10-21 BPL	**	**	***	**			**	***
3 - 03-16 BWR	*	***	*	**			**	***
3 - 5-27 CGG	***							
3 -18-25 CCR	***	*	*	*		**		
3 - 20-28 FRD	***	**	***	**	***			
3 - 22-18 IPN	***	*	**		***			
3 - 18-19 OCR	**	**	***	***	***	**		
3 - 10-30 PGI	***		**		***			
3 - 15-17 TCL	**	**		***	***			**

3.1.4. Time lapse determination of predictive active cell populations

While Chapter 3.2 primarily address reaction recognition and interpretation, the first reaction recognized (commonly a phase 2 reaction) trigger a measurement of the time lapse. Time lapse is defined as that length of time that occurs between the start of incubation and the specific time that the first reaction was recognized and recorded. Time lapse can be measured in days, hours or seconds and the interpretation as a predicted active cell population can be determined using: (1) the certificate of analysis which accompanies each box of testers; (2) the entry of the time lapse into QuickPop v 4 software (DBI) which 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); or (3) using the full video BART reader (VBR) system that allows the digital storage of time lapsed images of the BART testers over the time that the tests are incubated. The VBR system is suitable for the IRB-BART tester and recognizes the phases 2, 3 and 4 reactions and allows the saving and interpretation of the data entered.

The generation of the relationship between time lapses and the predicted population is made more challenging by the fact that natural samples are automatically complexed communities (consorms) in which individual species of IRB are integrated. Here the consormial activity may be of such a

type that individual species may be supplanted if it favours some particular activity. Relating the time lapse to a predicted population therefore is challenged by the ongoing on-going shifting of activities within the consorm. The approach used differs with the BART tester type. For the IRB- BART tester, a blended approach was employed to generate a best fit analysis that would then be used to generate an acceptable fit. For this eight pure bacterial cultures (Table 3.1.4.1) and three natural samples were employed. Note that for some of these cultures, tests will shift from one reaction type to another as the growth in the IRB-BART matures. For example, *Citrobacter freundii* may cause after 5 to 8 days a bio locking of the ball so that when the test vial is turned upside down the ball remains "glued" into position with the liquid medium held above the ball. The first reaction normally precedes the second reaction. The test using *E.coli* is performed at 35°C.

Table 3.1.4.1 IRB pure cultures and consorms used to project time lapse to population linkages

ATCC	Genus/species	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
25922	*Escherichia coli*	FO

For the eight ATCC strains the links were determined by culturing on quarter strength brain-heart infusion agar, washing of the colonies after three days incubation at 30±1°C (except *E. coli* which was incubated at 37±1°C) using sterile ringers solution. These concentrated cells suspensions were then subjected in a series of tenfold dilution using Ringers down to 10^{-9}. Each dilution was then inoculated into an IRB-BART inner tester (15ml suspension) and incubated at room temperature (22±2°C) and observed daily for the generation of the reaction as specified in table 3.1.4.1. Concurrently populations in the original cell suspension were enumerated using dilutions and quarter strength brain-heart infusion agar spreadplate colony enumeration with thirty to three hundred countable colonies being acceptable for calculation of the cell populations. For the three natural samples one ochre (3 – 18-19) and two ferric-rich plugs (3 – 19-21) were used extricated 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- inner tester (without media and BART ball). After mixing for thirty seconds the total 15ml was moved and dispensed into a regular IRB- laboratory tester. A serial dilution was now made using tenfold dilution with 1.5ml transfers made up with 13.5ml of Ringers solution into fresh sterile IRB- BART 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:

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

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 3.1.4.2 Note that populations are expressed as predicted active cells per ml (pac/ml) based upon daily observations being inputted into QuickPop version 4. 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 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 system (version 4) which can achieve acceptable precision. Intervals commonly employed for the VBR generation of time lapse data is 15minutes.

Table 3.1.4.2 Daily Observations of the IRB- BART tester showing variability in Predicted Populations

days:	1	2	3	4
high population	1,990,000	494,000	122,000	30,000
average population	598,000	140,000	34,800	8,630
low population	161,000	40,000	9,900	2,450

days:	5	6	7	8
high population	7,500	1,850	460	114
average population	2,130	529	131	32
low population	608	150	37	9

3.2. Sulfate Reducing Bacteria (SRB- BART) – Black Cap

Parameter code: sulfide biotester

Sulfate reducing bacteria (SRB) are a group of anaerobic bacteria that, as a part of their normal activities, generate hydrogen sulfide (H_2S). This product can cause a number of significant problems in water. These range from "rotten egg" odors, through to the blackening of equipment, waters and slime formations, and the initiation of corrosive processes. Detection of these microorganisms is made more challenging because they are anaerobic and tend to grow deep within the biomass as a part of the microbial community (consorm). Detection of the SRB is therefore made difficult because SRB may not be present in the free-flowing water over the site of the fouling but are growing deeper down in the biomass. Because of this, the symptoms of SRB fouling may precede their detection using the SRB- testers unless a successful attempt is made to disrupt these biofilms and cause the SRB to come out 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 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. They are defined as:

> "All bacteria that reduce sulfate or 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; or (2) the degradation of sulfur amino acids in proteins. Traditionally it has been considered that the H_2S originated solely from sulfate reduction but, in practice, samples having significant protein content could also generate H_2S during reductive protein degradation. 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 practise to use alloys incorporating iron in the structures (for example, mild steel in gas, oil and water pipelines). If these alloys become coated with biomass then there is a potential for reductive conditions to become established at steel: biomass interface which could then lead to the generation of H_2S. It is generally believed that H_2S is a major trigger for electrolytic corrosion particularly at anodic sites where biomass tends to actively congregate. 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 coatings. The cost of these corrosion controls to microbiologically influenced corrosion (M.I.C.) is very high and, generally, SRB are monitored as the prime indicator of M.I.C. risk. However another major component in M.I.C. is acidulolytic corrosion caused by acid producing bacteria (see APB, Chapter 4.5).

To achieve selective detection of the H_2S generating bacteria including sulfate reducers and reductive proteolytic generators the selective medium incorporates sodium lactate, sulfate, and yeast extract along with the inorganic

macronutrients. When prepared the sterile dried pellet weighs 241.2±1.3mg per tester.

3.2.2. Reaction patterns for the SRB- BART

There are two significant reaction patterns that can be interpreted as positive for the SRB- tester. 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 SRB populations the whole tester can go black. This is called black all (BA) but is not recognized since it is a terminal reaction and was 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 test vial. Blackening frequently starts as a 2 to 3-mm 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 the there is either the formation of a completely jet black cone in the base of the tester or when black bands appear at the bottom of the walls just above the conical base.

- Blackening at the top around the Ball (BT) involves the formation of a slime ring may be viewed around 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 banding. A positive is declared when granular jet black (Pantone black 6) granules appear in at least one sector 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.

It is recommended that no attempt be made to interpret any other forms of growth in the SRB- BART tester. These may be seen by the appearance of cloud-like structures in the colorless liquid medium or the formation of turbidity (clouding). Usually these structures form from the bottom up after which the clouding 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 fermentors but these reactions are not recognized sine they do not relate to SRB.

3.2.3. Bacterial consorms recognized by reaction signatures in the SRB- BART

There are only two reactions recognized for the SRB- BART 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 commonly difficult to control;
- BT reactions indicate that there is a dominant aerobic slime forming heterotrophic biomass which has active SRB present in the consorm. This reaction is more likely to be associated with the generation of

hydrogen sulfide from reductive proteolytic functions.

There was a third reaction (black all, BA) which does occur in the SRB- tester when the blackening appears up the length of the tester. When observations are made daily in a very active SRB population then the BA may be the first positive reaction seen. This may have arisen from a transient BB reaction (not observed) in which there was a dominant anaerobic consortium including SRB but with a significant fraction able to function within aerobic slime formers. If the aerobic slime formers incorporate SRB in the growing biomass around the ball then the SRB would be able to colonize under those anaerobic conditions generating a BT (not observed) reaction. Using the VBR digital time lapse photographic methods it should be possible to differentiate the occurrence of BB and BT reactions before the all-encompassing BA reaction occurs. Where a BA reaction is first observed the default would be to consider that reaction to have started as a BB.

Unlike the IRB- tester that has a complex set of interactions the SRB- tester is simple since it uses only one of two reactions (BB or BT) to differentiate the consorms containing detected SRB activity. In general the BT reaction will be generated in samples where the ORP 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 could generate hydrogen sulfide utilizing either the sulfates or 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 (e.g. 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 SRB 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- BART 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 recommended in *Recommended Practise for Biological analysis of Subsurface Injection Waters,* Volume 38, 2nd edition 1965. For the natural sample the primary effluent from an aerobic wastewater treatment plant may be used. 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.

3.2.4. Time lapse determination of predictive active cell populations

Both the culture of DSM1924 and the primary effluent were used as the sources for SRB to generate the relationship between population and time lapse using the VBR system. To achieve these serial dilutions were now made using tenfold dilution with 1.5ml transfers made up with 13.5ml of Ringers solution into fresh sterile SRB- BART 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 SRB in the sample. Agar spreadplate enumeration were included initially but tended to show a much lower sensitivity to the detection of SRB colonies than was obtained using the serial dilution method.

Regression analysis of the cumulated data generated equation two gave the most suitable correlation:

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

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 3.2.4.2

Table 3.2.4.2, Daily Observations of the SRB- BART tester showing variability in Predicted Populations (pac/ml)

days:	1	2	3	4
high population	8,180,000	1,880,000	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

3.3. Slime Forming Bacteria (SLYM- BART) – Lime Green Cap

Parameter code: slime biotester

3.3.1. 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. These slime-like growths are therefore often not dominated by the yellows, reds and browns commonly seen where IRB are present. Some of the IRB also produce slime but it is sometimes denser and has more texture due to the accumulation of various forms of insoluble iron. SLYM bacteria can also function under different reduction-oxidation conditions but generally produce the thickest slime formations under aerobic (oxidative) conditions. These can develop in the SLYM- tester as slime rings growing around the floating ball. Slime growth can also be seen as a cloudy (fluffy or tight plate-like structures) or as gel-like growths which may be localized or occur generally through the body of water medium. Very commonly the gel-like slime growths form from the bottom up in the test vials. One common check for these types of growth is to tilt the BART™ gently and see that the cloud- or gel- like growths retain their structure and tilt with the tube. 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 remain active while being shielded by the bound water"

Many bacteria can produce slime-like forms of growth when they generate biofilms which retain water. Bonded water is actually formed by a variety of exopolysaccharide polymers substances (EPS) that are long thread like molecules. These forms of 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. Some bacteria produce an EPS that remains tightly bound to the individual cell. These are called capsules. Other bacteria generate such a copious amount of EPS that it envelops whole masses of cells within a common slime. The role of the slime appears to be protective. If environmental conditions are harsh (e.g., due to shortage of nutrients), the slime layers tend to 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 nutrient base, or otherwise be toxic to the cells. EPS may be produced by enzymatic activity (e.g., dextran sucrase or levan sucrase) on carbohydrates. In addition, EPS may be synthesized within the bacterial cells and released to form an enveloping slime.

To achieve slime formation the medium used is composed mainly of proteose and peptone-tryptone. 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- BART 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 with sterile water is added then there is a dissolution and diffusion of the proteose and peptone-tryptone dried pellet vertically into the water column. This generates a concentration diffusion gradient that also triggers the oxidative-reductive interface to form and move upwards at the same time. This can create a focus of activity within the elevating interface and diffusion fronts. This focus can trigger early visible reactions when the slime forming

bacteria form as zones of growth often elevating with the fronts.

3.3.2. Reaction patterns for the SLYM- BART tester

There are eight reactions recognized for the SLYM- BART tester:

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). Descriptions of the various reactions are given below:

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

- Cloudy Plates Layering – CP is a reaction that can occur when there are populations of aerobic bacteria. Here the initial growth may be at the oxidative-reductive interface that commonly forms above the yellowish-brown pellet diffusion front. This growth usually takes the form of lateral or "puffy" clouding which is most commonly grey in color. Often the lateral clouds may be disc-like plates that are 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 tube. These formations are most commonly observed 10 to 25 mm beneath the floating BART ball. Sometimes these plates will appear to divide laterally to form multiple plating. CP reactions usually terminate by 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 BART ball beneath the water-headspace air 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 very cloudy.

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

- Thread-Like Strands – TH occur 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 BART ball.

There are ranges of reaction pattern signatures (RPS) for the SLYM- BART 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;
- 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

usually between day three and five and then commonly fades;

- 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

3.3.3. Bacterial species recognized by reaction signatures in the SLYM- BART

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;

All of the above reactions utilizing quarter strength brain heart infusion agar spreadplates for the initial culture of the individual species followed by dispersion into sterile Ringers solution to obtain a cell population of 10^7 to 10^8cfu/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±2°C with the reactions being generated sequentially within four days.

3.3.4. Time lapse determination of predictive active cell populations

To determine the relationship between time lapse at 22±2°C and the cells populations inoculated into the tester, pure cultures of the bacterial species (see 3.3.3 above) were inoculated into HAB- 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 spreadplate technique employing 1.0ml of the diluant. Best fit 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:

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

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

days:	1	2	3	4
high population	7,510,000	1,470,000	288,000	56,500
average population	1,730,000	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

3.4.1. Heterotrophically Active Bacteria (HAB-BART) – Blue Cap

Parameter code: bacterial biotester

Some bacteria are able to degrade organics as their source of energy and carbon. These are known as heterotrophically active bacteria ("organic busters"). By far, the majority of these heterotrophs function most efficiently under aerobic (oxidative) 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 engineered operations. HAB-BART 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 samples blue under oxidative conditions and clear under reductive conditions. 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) than under reductive conditions (producing fatty acids). While there remains free oxygen in the water, 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) activity then the methylene blue shifts from its observable 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 4% then it would be necessary pre-dissolve the methylene blue in the cap using 1.0ml of sterile distilled water. There is a reductive color shift (blue to clear) which works effectively over a pH range from 2.5 to 14.0. Very acidic water will also cause the water to shift from blue to clear. When pH falls lower than 1.5 then there is automatically a clearing. Another interference factor that could cause absorption of the methylene blue is high concentrations

(>300ppm) of petroleum hydrocarbons. Definition of HAB- bacteria is summarized below:

> "Any heterotrophically active bacteria are defined by those bacteria that can generate an active biomass with the function being to degrade the organic materials in the sample by respiration (oxidatively) or fermentation (reductively). This activity is monitored using the shifting in the oxidation-reduction potential that is observed using methylene blue as the indicator"

When the active bacteria cause reductive conditions to develop then the blue color is bleached out. This usually occurs from the bottom (bottom up) for aerobic bacteria; or from the top (top down) 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 to allow the methylene blue chemical dried in the cap time to dissolve into the water. When the HAB- tester is returned to it's normal state (cap side up), wrist action rotation of the tester causes the ball to roll through the water sample causing the methylene blue to become mixed into the water to form an even blue solution and at the same time become saturated with headspace oxygen.

Methylene blue is a basic dye that can bind readily to the negatively charged microbial cells. Traditionally, therefore, this dye has been used to stain microbial cells. A feature of methylene blue is that it changes from a blue color in the oxidized state to a clear form in the reduced state. When methylene blue is added to a medium that is actively converting energy due to microbial respiration, the electrons

are transferred to the dye causing it to become reduced and the dye changes from a blue to a clear state (the color disappears). The protocol has been based on the methylene blue reductase test that has been used in the dairy industry for decades to determine the potential for bacterial spoilage of milk. In the HAB- 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 bacteria in the sample water is at an end.

To achieve the activity of the heterotrophic bacteria the selective culture medium 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 water sample or the porous semi-solid sample with sterile water is added then there is a dissolution and diffusion of the proteose and peptone-tryptone dried pellet vertically into the blue water column.

Note that when the HAB-BART tester was originally developed (1988 to 1993) it was considered that the tester was 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 a very effective method for the generalised detection of bacterial activity and the name was changed to “heterotrophically active bacteria” to recognize the anaerobic activity associated with this tester.

Unlike the other BART testers that operate solely at 22±2°C the HAB- tester can also be operated at 28±1°C but only for the determination of bacterial populations in municipal sanitary wastewaters. 28±1°C is optimal for maximizing the activity of bacteria in wastewater treatment plants and VBR version 5 has to be used since it is customised to that optimal temperature. The differences between VBR versions 4 and 5ww are discussed below in Chapters 3.4.3 and 3.4.4. For wastewaters the use of the higher optimal temperature speeds up the bacterial activity and allows a time lapse (and population) to be generated commonly in two to sixteen hours.

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 bromocresol 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) 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.

3.4.2. Reaction patterns for the HAB- BART

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

- 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 beyond the equator of the ball so that a blue ring will 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.

- DO reactions occur where the bleaching moves downwards from below the floating ball. 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.

3.4.2.1. Bacterial consorms recognized by reaction at $22 \pm 2^{o}C$ in the HAB- BART

Two ATCC bacterial strains 27853, *Pseudomonas aeruginosa;* and 25922, *Escherichia coli* were used to define the reactions UP and DO respectively. Spreadplate populations were obtained from these cultures and also from natural samples using 0.1ml dilutions in sterile Ringers solution down the 10^{-8} with streaking out on quarter strength brain heart infusion agar, incubation at $22 \pm 2^{o}C$ 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 $22 \pm 2^{o}C$. One third of the natural samples came from reductive environments (giving DO reactions) while the remainder came from oxidative environments (giving UP reactions)

3.4.2.2. Bacterial consorms recognized by reaction at $28 \pm 1^{o}C$ in the HAB- BART

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 spreadplates with three replicates for the HAB-BART 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 spreadplate enumeration trials it was found that the pac/ml generated from the VBR version 5ww matched with the colony forming units that were generated by the traditional technique.

3.4.2.3.Time lapse determination of predictive active cell populations, HAB- BART (22±2°C), VBR version 4

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

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

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 3.4.4.1.)

Table 3.4.4.1, Daily Observations of the HAB- BART tester showing variability in Predicted Populations (pac/ml)

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

3.4.2.4. Time lapse determination of predictive active cell populations, HAB- BART (28±1°C) for VBR version 5.

There are two equations for the operation of the HAB-BART testers incubated at 28±1°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, five is used where the time lapse (x) in seconds is <86,401 and six is employed when x is >86,400. Table 3.4.4.2 shows the relationship between time lapse (x, seconds) and the calculated predicted populations (pac/ml) using both equations Note that in the next table the .Note: populations are in increments of 1,000, this table shows hours for the time lapses assuming VBR is used with digital images of potential reactions every fifteen minutes; the upper table uses equation five and the lower table (*) uses equation six. High and low populations are calculated from time lapse using the 15 minute intervals between images and so deducting 899 seconds for the high and adding 899seconds for the low population to give the range. Shortening the intervals to less than 900 seconds would increase the precision.

Table 3.4.4.2, Daily Observations of the HAB- BART tester showing variability in Predicted Populations (thousands pac/ml) using 28±1°C with observational readings in hours.

hours:	4	8	16	24
high population	83,100,000	15,800,000	3,010,000	109,000
av. population	23,900,000	4,570,000	165,000	6,020
low population	6,910,000	251,000	9,120	36.6

hours:	32	40	48	64
high population	724	13.5	0.253	0.004
av. population	22.2	0.416	0.007	0.001
low population	0.685	0.012	0.001	0.001

3.5.1. Acid Producing Bacteria (APB- tester) - Purple Cap

Parameter code: acidogenic biotester

There are two major groups of acid producing bacteria that are most commonly associated with 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 which would not be detected by the APB- tester. It is the (organic) acid producing bacteria (APB-) that are detected by this tester. These APB cause the pH to drop significantly from neutral to acidic conditions ranging from terminal pH levels from 3.5 to 5.5. APB activities are formed by a variety of heterotrophic bacteria that share the common ability to produce organic acidic products when growing under reductive conditions utilizing organics (fermentation). These mildly acidic products are sufficiently corrosive impact the integrity of many metallic alloys (e.g. those with a high aluminum component). Because of 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 the 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. SRB corrosion event occur primarily under highly reductive conditions in the presence of adequate sulfates and organics. In general the SRB generated hydrogen sulfide (H_2S) as a metabolic

product and it is this product that then triggers the electrolytic corrosive processes. Some MIC occurs under mildly acidulolytic conditions. Acidulolytic corrosion appears to be caused by bacteria able to generate acid products generally under highly organic and reductive conditions. Today it is recognized that the APB are significant contributors to corrosive MIC processes. In these cases then the compromises is likely to be through the gradual dissolution of the metal under the very acidic conditions that are created (e.g. shallow lateral dishing of steel walls and gradual losses in strength and 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 the metal surface is 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 growth and the metal surface. This is different to the electrolytic corrosion caused by SRB which tends to cause deeper pitting of the metals and deeper 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 cavitation within the metal.

To generate acid conditions from the production of the shorter chained fatty acids, the selective culture medium pellet is rich in proteins and glycerol. An individual pellet is composed on tryptone, peptone, and glycerol along with the basic macro-nutrients and weights 484±3.0mg. 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. It is recommended that samples 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.

3.5.2. Reaction patterns for the APB- BART tester

There is a single reaction pattern recognized for the APB- tester and that is caused by the pH indicator, bromocresol purple, shifting 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 that the reaction should be considered as positive. For many samples being tested the APB within the sample may react to buffer out the pH and this buffering effect would mean that the tester would return from DY to a shade of purple. When this occurs it is more common to see the buffering extending from the bottom upwards, top downwards or as a series of expanding patches. For the APB- BART tester it is therefore very important to monitor the test sufficiently frequently (e.g. every six hours, use the VBR system). Buffering is not a recognized reaction for APB.

3.5.3. Bacterial reaction signatures in the APB- BART 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 facultative or strictly anaerobes which have the ability to degrade a multiplicity of organics with 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 not recognized. 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 buffering occurs. It is therefore important to minimally observe the test daily for reactions or use the VBR system. For the ubiquitous fermenting bacteria under reductive conditions normally will generate some level of acidic product. 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#: 8090, *Citrobacter freundii*; 23355, *Enterobacter cloacae*; and 13315, *Proteus vulgaris*.

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

Due to the wide abundance of APB in the reductive environments the correlation between time lapse and population was prepared using natural samples. Populations were assessed using serial dilutions of the samples to 10^{-9} using 15ml of sterile Ringers as the diluant and 1.5ml as the transferred volume between diluents. All 15ml of each completed diluant was then transferred to an APB- tester which was then incubated for ten days at room temperature (22±2°C) and observed for the time lapse to the generation of a DY 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 where 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 spreadplate 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:

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

In equation one 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 3.5.4.1.

Table 3.5.4.1 Relationship between time lapses (days) and predicted population (pac/ml) of APB

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

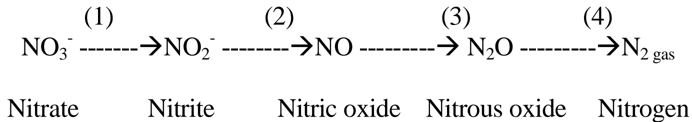
3.6.1. Denitrifying Bacteria (DN- BART) – Grey Cap

Parameter code: nitrite biotester

DN is short for denitrification (reduction of nitrate). This activity is extremely important not only in environmental but also in geochemical terms. The reason for this is that the essentially all of the atmospheric nitrogen (N_2) has been derived from the process of denitrification which is driven by the denitrifying bacteria. It is therefore an extremely important stage in the nitrogen cycle in the crust of planet Earth. There is a distinctive cycle in which nitrogen from the atmosphere is fixed, cycles through the biomass, is oxidized to nitrate by nitrification (see N-BART, 3.7.1.) and is finally reduced back to nitrogen gas by denitrification which is controlled by the denitrifying bacteria. Denitrifying bacteria are therefore an important environmental indicator group for the decomposition of waste organic nitrogenous materials. These 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. Such waters are most likely anaerobic (free of oxygen) and relatively rich in organic matter.

A common use for the presence of aggressive denitrifying bacteria in waters is that these bacteria signal the 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. 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.

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:



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

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

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

3.6.2. Reaction patterns for the DN- BART

Denitrification as a recognizable is achieved when the nitrate is reduced through to nitrogen gas that then collects as a foam ring around the BART ball.

FO - Foam around floating BART ball

There is only one reaction recognized in the DN – tester that occurs only when the nitrate is completely denitrified to nitrogen gas that then collects as a detectable foam (interconnected gas bubbles) ring around the BART ball. Casual presence of gas bubbles attached to the side walls of the tester or on the underside of the ball should be ignored since these bubbles could relate to the transient presence of either gases such a 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.

Solutions in the tester usually go cloudy but this should be ignored and the major positive for FO is the presence of very many interconnected gas bubbles forming foam as a continuous ring around the ball. This shows that complete denitrification has occurred and the denitrifying bacteria are present. It should be noted that the foam ring may consist of as little as a single ring of bubbles to a thick foam as much as 5mm high commonly originating at the equator and going upwards. This thick foam commonly last for one to two days and, on rare occasions, can be seen to support bacterial biomass growing above the foam (e.g. iron oxidizing bacteria producing ferric-rich plates).

3.6.3. Bacterial activities recognized by denitrification in the DN- BART

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 four species give the foam ring after two to three days of incubation at room temperature or (28±1°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. Final effluent from aerobic municipal wastewater treatment plants can be substituted to obtain denitrification using the DN- BART.

3.6.4. Time lapse determination of predictive active cell populations

Due to the wide abundance of DN in the reductive environments the correlation between time lapse and population was prepared using natural samples. Populations were assessed using serial dilutions of the samples to 10^{-9} using 15ml of 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°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 spreadplate 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 one gave the most suitable correlation:

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

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 3.6.4.1

Table 3.6.4.1. Relationship between time lapses (days) and predicted population (pac/ml) of DN

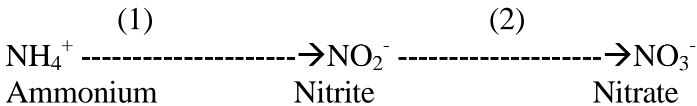
days:	**1**	**2**	**3**	**4**
high population	12,500,000	1,460,000	172,000	20,200
average population	1,810,000	213,000	25,100	2,940
low population	264,000	31,000	3,650	429

days:	5	6	7	8
high population	23,800	279	32	3
average population	346	40	4	1
low population	50	5	1	1

3.7.1. Nitrifying Bacteria (N- BART) – White Cap

Parameter code: nitrate biotester

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



Nitrifying bacteria are divided 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 is a polarized relationship between the nitrifying and the denitrifying bacteria. Here oxidative conditions will trigger nitrification and reductive conditions will favor denitrification. This becomes a problem when testing natural samples since the two groups are either producing or utilizing nitrate respectively. In developing a biodetection system for the nitrifying bacteria in natural samples, the terminal product (nitrate) may not be recoverable as an indicator because of the intrinsic activities of the denitrifying bacteria which are also likely to be present and active in the sample. It is because of this difficulty that the N- tester restricts itself to detecting the nitrosofiers that generate nitrite. This nitrite will be oxidized to nitrate by the nitrifiers only to reappear again when reduced back to nitrite when any intrinsic denitrification activity occurs in the culturing sample of 7.5ml.

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 when aerobic (oxidative). Nitrates in water are a cause of concern because of the potential health risk particularly to infants who have not yet developed a tolerance to nitrates. In soils, nitrification is considered to be a very significant and useful function in the recycling of nitrogen through the soil. Nitrate is a highly mobile ion in the soil and will move (diffuse) relatively quickly while ammonium 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 sequence of each other. It is recommended that, where activity is determined then such waters should be subjected to further evaluation as a hygiene risk through a subsequent determination for the presence of nitrates and resulting 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 test detects the nitrifying bacteria that are able to oxidize ammonium (NH_4) to nitrite (NO_2) and on to nitrate

(NO_3). This test uses a selective medium for the bacteria able to oxidize ammonium to nitrite by examining chemically for the nitrite product. The additional two balls used in this test 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 is used to detect the presence of nitrite that is generated during the early stages of nitrification. If the sample being tested also contains denitrifying bacteria, nitrite may again be created by the reduction of nitrate (denitrification). This test method has been developed in consideration of the greater likelihood of nitrite being detectable rather than the (product) nitrate. Note that this test cannot function in water samples with a natural nitrite level of greater than 3.0 ppm. Water samples with greater than 28 ppm of nitrite will automatically turn the liquid medium to a yellow color when the reaction cap test is applied.

In the N- BART tester there is a total dried chemical component with the pellet and the reaction cap of 209±3.0mg per tester. The selective culture was based on ammonium sulfate mineral salts and the reaction cap detected the presence of nitrite.

3.7.2. Reaction patterns for the N- BART

This tester involves an unusual BART test in that the presence of nitrifying bacteria is detected after a fixed incubation period of five days. Positive detection is therefore achieved using the presence 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 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 BART balls and in solution. There are three recognized levels of positive detection of nitrite as an indicator of nitrification (Table 3.7.2.1.):

Table 3.7.2.1 Reaction patterns for positive denitrification in N- BART testers

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

Notes: * 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: (PB) relatively inactive population of nitrifiers (< 10^2 nitrifiers/ml) associated with aerobic slime forming bacteria in a consortium; (RB) 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. Where the sample does contain nitrates and denitrifying bacteria are active then a positive detection for nitrite can be found where denitrifiers are active. This represents a false positive but only occurs when the sample being tested already contains nitrate at greater than 0.5ppm.

3.7.3. 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.

3.7.4. 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 that there needs to be greater precision in the prediction of the active nitrifying bacterial population then a serial dilution of the liquid sample can be employed. 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 not 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.

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

Parameter code: microalgae biotester

Green 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 (at the light threshold point where the static water level occurs means that these green blooms are not so evident and so generally ignored. The designation of ALGE-tester examines for the presence of active micro-algae in the sample is the name given to various plant-like microorganisms that 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 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- tester uses a modified the Bold medium which does not contain organics, but does contain the basic nutrients for plant growth (nitrogen, phosphorus, potassium, sulfur etc.). Carbon is presented in the tester as bicarbonates while 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 it does require the presence of light (for photosynthesis). Most micro-algae can actually utilize quite low levels of light for growth. For example the charged ALGE- tester can be placed on its side and set it about 60 cms from a single 40 watt daylight fluorescent light. 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. 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 Chapter 3.8.2). Observations can determine:

(1) level of activity of the micro-algae (activity) through the time lapse before a reaction is 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

3.8.2. Reaction patterns for the ALGE- BART tester

There are therefore six reactions that can be observed in the ALGE- BART tester as shown in Table 3.8.2.1. 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 3.8.2.1 Six reactions commonly seen in the ALGE-BART tester

	Growth	Description	Pantone
GG	Grass Green growth	A grass-green growth may be seen through the porous textile medium usually concentrated at the water line or below water line. As this reaction matures, flocculent green growth may also be observed in the liquid medium.	555
FG	Fuzzy Green Patches	Much of the reaction seen here may be above the water line in the semi-saturated porous textile medium. The pore structure restricts the extension of the growth and so the growth may be seen as intense grass green zones with ill-defined or radial edges.	555
OB	Red Orange Brown Patches	Red, orange or brown patches usually with clearly defined edges are generated both above and below the water line. These growths may gradually change in color as the growths mature.	7425 715 7524 7525

	Growth	Description	Pantone
YB	Yellow Beige Patches	Poorly defined light yellow to beige patches of growth occur on the fabric often at localized sites on the porous fabric, generally these growths are initially difficult to observe.	7404 728
GF	Green Flocculent	Grass-green flocculent deposits are abundant in the liquid medium and on the floor of test vial. Tendency to be dense and lay on the floor of the test vial. The porous fabric may also show some green discoloration particularly on the lower side.	554 555
DG	Dark Green to Black Patches	Predominantly recognized by dark-green, blue-green or black growths at the water line. Often this is a secondary reaction following reactions GG, FG or GF	582 567 5535

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°F (30°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 in the test device. 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 a number of 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 a Bold 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

In summary, the colors present by the six different reactions do occur at different sites in the ALGE- BART 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.

3.8.3. Micro-algal communities recognized by reaction signatures in the ALGE- BART 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 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.

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

Challenges to the ALGE- BART tester as a semi quantitative measure of the numbers of micro-algae in the sample are 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 eight gave the most suitable correlation:

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

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 3.8.4.1)

Table 3.8.4.1. Relationship between time lapse (days) and predicted population (pac/ml) of micro-algae using the ALGE- BART 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	2,520

days:	16	24	32
high population	1,100	53	2
average population	783	38	1
low population	558	27	1

Chapter 4

Factors Affecting BART Testing

4.1. Significant Factors that can affect the BART testing procedures

While the BART testers can yield valuable information regarding the microbiological composition of the sample being tested, there are numbers of important considerations that need to be addressed. Chapter 5 utilizes the Quick-Break Training to discuss different aspects of this that have been raised by the BART users.

4.2. Sampling

The success in analysis of a given water sample is dependent upon the validity of the sample taken as being typical of the site being investigation. Any water sample when taken is composed of suspended (planktonic), particulate (mainly in biocolloidal forms), along with sheered materials coming from attached bacterial biomass fouling surfaces, pores and fractures present or connected to the site being sampled. Sampling from a site where there is no disruption then it is probable but not certain that the majority of the bacteria detected would have come from planktonic and biocolloidal sources but not attached. Since greater than 80% of the bacterial biomass is attached to surfaces then these would not be accounted for in the analysis of that sample. To recover these attached bacteria then there has to be some shock applied to the sampling site prior to taking the sample. Such a shock can be relatively simple since changing the environmental conditions (turn off the pumps for a day or dead end the lines) to cause at least some of the attached bacteria to release from the surface and enter the water where they can now be sampled.

Porous and fractured media (e.g. soils, filter media, encrustations) present a different challenge since different bacteria will be active at different points within the environment. Major factors here would be the bound water content, charges on surfaces, size of pores and any fractures; and the availability of nutrient and toxic chemicals. These media are therefore more challenging since any bacterial activities are more tightly defined.

Assuming an acceptable sample has been taken then the tester provides a sensitive method for the detection of bacteria. It might be an advantage to examine for bacterial activity in the sample using the ATP technique (Chapter 5.13). If little or no ATP is detected then there may be no value in applying testers to such "dead" samples. Testers come in two formats that make them easy to be used in field or laboratory conditions. The major differences between the field and laboratory version of the tester is that the field tester has a second vial (bottle) that provides additional stability and protection to the tester when it is being transported and used in the field. Laboratory versions are more economical involving only the inner vial (bottle) and are designed to be tested in the laboratory setting using test tube racks to ensure they stay upright. Field versions involve the second outer vial (bottle) to provide additional protection to the inner vial. There are occasions when there is a need to take a water sample that would then be used to fill the inner vials (testers) while out in the field With the field testers there is the potential to use the outer vial (bottle) as the means of collecting the water sample for use in the testers. The inside contents of the field tester are sterile and so therefore, when removed, the outer vial remains effectively sterile and can be used to collect the water sample. To do this use the following procedure: (1) unscrew and remove the outer cap, remove the inner tester and place in the aluminum foil pouch from which the tester was taken, and lay the outer cap down on a clean surface without

turning it over; (2) screw the outer cap back onto the outer vial and it is now ready to be used for collecting the water sample; (3) when collecting the water sample then remove the outer cap again and place on a clean surface; (4) add the water sample to the outer vial but do not fill beyond the fill line beneath the threads, this line denotes that 65ml of water has been added; and (5) put outer cap back on to the outer vial and screw down. Up to 65ml of water sample can be taken using one outer vial. This would be enough to charge four inner testers. It should be remembered that the water sample only remains valid if it has not been contaminated during collection. Therefore do not charge the outer vial in an environment that is dust laden and always handle the outer vial from the outside to avoid contaminating the inside of the sampling bottle. If sterile latex gloves are available then it is advantageous to handle the outer vial wearing thee gloves to further reduce the risk of contamination. There are no chemicals added to the outer vial and so any chemicals present in the water (for example, chlorine) would not be neutralised. However all testers do include sodium thiosulfate in the inner vials and so any chlorine impacts on the bacteria in the sample are limited to that period of time before the samples are dispensed into the inner testers. It should be noted that all BART testers have to pass through a rigorous ISO 9001:2000 certification process that includes sterility checks, the use of clean rooms to minimise contamination and full quality management procedures to ensure that the products meet all claims. All sampling procedures need to be followed in both the taking and the subsequent storage of the water sample prior to starting the testers. See Chapter 4.6 for more details on the storage of water samples if there is some delay before starting the tests. Read "Certificate of Analysis" which accompanies every box of BART testers for the protocol to set up the test.

4.3. Oxidation-Reduction Potential (ORP) implications from BART tester reactions

IRB	CL	+200 to -100
	FO	-20 to -150
	BR	+200 to +10
	BC	+50 to -50
	BG	+10 to -50
	RC	0 to -50
	GC	+50 to -20
SRB	BT	+50 to -20
	BB	-20 to -150
SLYM	DS	+20 to -100
	SR	+200 to +10
	CP	+50 to 0
	CL	+200 to -100
	BL	-20 to -150
	TH	+100 to 0
	PB	+200 to +10
	GY	+200 to +10
HAB	UP	+200 to -10
	DO	-10 to -150
APB	DY	0 to -150
DN	FO	+5 to -150
N	PP	+200 to +10
	RP	+200 to +10
	DR	+200 to +10
ALGE	GG	+200 to +20
	FG	+200 to +10
	OB	+100 to 0
	YB	+200 to -10
	GF	+200 to +10
	DG	+50 to -20

Table 4.3.1 ORP ranges (mv) for the major reaction patterns (see page 80 for table).

The ORP generated in the BART tester is a combination of the ORP value found in the sample and the influence that this has had on the charges associated with the different reactions (Table 4.3.1. above). Note that the ORP ranges (in millivolts, mv) are given (right hand column) for the established reaction (center column) generated from the BART tester (left hand column). These are presented for each reaction is the common range observed and it has to be remembered that the reaction being observed in the active biomass will involve gradients. Hence the ORP values are shown as ranges. ORP is an expression of the electrical charges within the sample expressed in millivolts (mv). Oxidative conditions have a positive mv while reductive conditions are negative. Essentially oxygen is present in +mv and hydrogen in –mv as an expression of the electrolysis of water within that environment. Remember that, with a microbial biomass, there can be significant ORP gradients within that biomass that may affect the bacterial activities.

4.4. Visual BART reader (VBR) test system

From the beginning of commercial use of the BART testers there has always been a frustration with getting an accurate time lapse for significant reactions when daily observations were conducted. Technology has gone in leaps and bounds since 1990 and DBI kept improving and developing new reader systems to automatically detect reactions and accurately record time lapses. These readers did generate precision but not reliability and so none were released beyond beta trials. From 2006 it was realised that the use of complex photo-electronics was too uncertain to be economical and reliable. At this time came the development

of simple digital time lapse photographic methods that coupled seamlessly to computers. The visual BART reader (VBR) system evolved out of these technologies and the challenge became examining simultaneously the reactions being generated by the many various testers. Limitations became the use of room temperature (22±2°C) and background room lighting from daylight fluorescent diffuse lighting. Advantages are that it is possible to use the VBR system for the following testers: IRB-, SRB-, SLYM-, HAB-, DN-, APB-, and (in a protracted sense) the ALGE-BART. Only the laboratory versions of the BART testers can be used in the VBR system.

For the camera it was found that one of the more basic Cannon cameras would meet the needs and would utilize the Cannon software for time lapse photography. The selected camera has automatic focus which means that the focal plane is set on the forward edge of the tester. DBI has developed software that integrates with the Cannon software and allows digital pictures of the testers paced in the racks set for selected timed intervals such as 5, 15 and 30 minutes with automatic interpretation of recognized reactions into populations (pac/ml). VBR version 4 software allows the user to scroll through all of the recorded images (like a movie) and select the frames where different reactions first occur and all information generated by the software is archived at the operator's initiative. At this time there is only one condition where the VBR system can be used at an incubating temperature different to room. That is for municipal sanitary wastewater where the incubating temperature is 28±1°C (see also 3.4.4.2). This has been done in VBR version 5 to optimise the temperature to that which appears to support the maximum amount of bacterial and, hence, the fastest and most precise delivery of the data.

From Beta trials it has become evident that the optimal size for the VBR rack would be 24" (w) x 24" (h) x 8" (deep)

and would hold thirty laboratory testers. Both VBR versions 4.0 and 5.0 utilize the software supplied by Cannon which allows time lapse photography to be taken for the BART testers in the VBR rack (maximum 30). Once the camera is connected through the USB port and the software installed then it is possible to interpret and archive the data and also generate reports in word (rtf) or add the data to spreadsheets. Additionally it is possible to take .jpg or .bmp images of selected testers in any frame. Setting up the DBI software involves using a computer that is Windows 95 or better including Vista. This software controls the camera by being compatible with the controls built in by Cannon. Once started it is possible to start lab BART testing at different times and also start new tests in rack slots once the old tester runs has been terminated in that slot. It should be noted that the VBR version 4 system functions at room temperature ($22\pm2^{o}C$) under continuous diffuse lighting away from any direct sunlight. It is recommended that the VBR rack system be kept in a position that allows easy replacement of testers and away from casual shadows thrown by people moving through the room as well as the effects of direct sunlight (changing the light intensity and heating the testers). VBR version 5 operates at similar locations to version 4 but involves the necessary heating pad and sensors to operate at ($28\pm1^{o}C$). Version 5 is recommended for use with sanitary municipal wastewaters and generally a run is completed in less than 20 hours.

Viewing the reactions can be helped by the use of the zoom and drag features in the VBR software. Zoom allows the observer to move into a particular group or an individual tester and drag allow you to move over the whole field of (potentially 30) testers. Positive detections are achieved by clicking on the reaction observed (each tester type has different drop-down reactions and they are all listed appropriately. When assured that the reaction is correct then clicking the confirmation button which causes the time lapse

to be calculated for that frame on-screen along with the predicted active cells per ml (pac/ml) if this is the first reaction observed in the tester. Each rack is equipped with two black lines along the back of the rack that allows the early detection of cloud reactions. Subsequent reactions are archived and presented in the interpretation but do not play a role in predicting the population. VBR version 4 is designed for use with the SRB-, IRB-, SLYM, HAB-, APB- and DN-BART laboratory testers only. For more information then go to the site "Visual-Bart-Read" or VBR at www.dbi.ca.

4.5. Biofilms, Biomass and BART terminology

Two common terms used to describe microbial activity are biofilm and biomass. Biofilm growths are defined as being attached films of microbial growth on surfaces in which much of the water is bound. As biofilms develop they go through a number of changes: (1) stratification with reductive environments underpinning the oxidative; (2) obsessive accumulation particularly of metallic cations and carbonates; and (3) decreasing porosity and increasing density. One common feature of all of these stages of biofilms is the presence of general heterotrophic bacteria and so the most useful tester would be the HAB-. It would allow the detection of aerobic oxidative bacteria as UP reactions and reductive anaerobic bacteria through reductive (DO) reactions. The activity level (population) may be determined by how fast the reactions occur (time lapse). Young biofilms would generally give UP reactions while fragmenting aging biofilms would give DO reactions sporadically.

Biomass is a common term applied to the total growth mass that has a defined site. Oak trees and humans both have a defined biomass. Microbial biomass is a little more difficult to assess as defined structures. The microbial biomass

associated with an oak tree is actually around the roots but forms a defined structure and even in the wood!. In humans the biggest active microbial biomass is actually in the intestine! Around inanimate objects like water wells then the defined biomass forms around that well as water is pumped into, or out of, the well. This biomass functions to support the microbes growing around the well but leads to changing water quality and commonly reducing flows. Sometimes a growing biomass can impact on surfaces to which it is attached. For example metal surfaces can corrode, lose strength and finally fail. Such negative effects can also be referred to as biofouling where there is a negative impact created by that biomass. Here corrosion is one of the most important economic factors generated by microbial biomass (see also 4.8).

If you want to examine the various bacteria present within a natural biofilm then the most likely bacterial communities (other than HAB) would be the sulfate reducing (SRB) and the iron related (IRB) bacteria. SRB- testers will define reductive and oxidative activity through the generation of a BB (black base) and a BT (black top) reaction respectively. Again QuickPop can be used to convert the time lapse to predicted active cells per ml (pac/ml). BB reactions can be expected deeper inside the biofilm while BT is more likely to occur on the outside of the biofilm. IRB- testers are the more challenging of the three to use simply because it is reactive to the iron oxidizing and iron reducing bacteria that may be commonly present in the biofilm in different layers. Most commonly the first reaction under reductive conditions is foam (FO) while for oxidizing conditions then the first reaction is clouding (CL). If the biofilm is forming ochre then the reaction seen first is a basal gel (BG) where a darkened green gel forms in the bottom of the tester.

4.6. Collecting and storage of water samples for BART testing

There is always a concern as to how long may water samples can be stored before beginning testing for bacterial activity. This concern stems from the fact that the sample was satisfactory is testing immediately but the sample begins to degrade as a result of the bacterial activities in the sample. For much of inorganic chemistry this is not an issue but when the objective is to examine the sample for bacterial activity then the longer the sample is kept before testing begins then the greater would be the potential impact These impacts stem from changes in the environmental conditions particularly temperature, oxygen content, and changes in turbulence. Temperature shifts can have a major impact since bacteria adapt to specific ranges associable with the source environment. Generally if the temperature shifts upwards or downwards by more then a couple of degrees (Celsius) then the bacterial community in the sample may respond. In either event the bacteria would be likely to shift in activity levels that could affect precision. Oxygen shifts are particularly critical in conditions where the oxygen levels are stressed (<1.4ppm O_2) pr technically absent (<0.04ppm O_2). Here the stress increases on the aerobic (oxidative) bacteria and decreases on the anaerobic (fermentative, reductive) bacteria. Net results of shifting oxygen levels can therefore be biasing in the community activities towards those favouring oxidative or reductive conditions. Turbulence is the third major factor that can affect the precision of bacteriological testing. Very commonly some bacteria will grow attached the surfaces. For these bacteria to be present in the sample then they must have sheered through turbulence from the biofilms into the water sample. Additionally bacteria within floating slime formations (biocolloids) may also break away due to turbulence. These factors mean that there is a probability that the sample contains more evidence of bacterial activity

(turbulence) or stress (temperature shifts or changing oxygen levels). The longer the sample is stored before analysis then the greater is the potential for variability. The common practise of placing a water sample over ice prior to testing can exaggerate these stresses but will slow down activity at the same time. Over ice brings the sample's temperature down to within the range from 0 to 8°C and is thought to "preserve" the sample for a longer period of time.

When out in the field collecting water samples for BART testing and you do not want to start the testers until you get back to the office, the challenge arises as to how you keep the water samples until you get back to a place when you can do the testing. Obviously keeping the water samples for longer than a day creates problems since the bacteria in the water sample will change in their activity levels and dominant communities over time. There can be no doubt that changes will occur in bacterial activity but to level the "playing" field all of the samples should go through a common protocol in which the storage time is reasonably consistent. It is well known that most bacteria go into a dormant state when temperatures are reduced below 7°C. This can be done by placing the water samples into a refrigerated environment (4±2°C) using a small portable refrigerator. Make sure that the sample bottles are not packed in too tightly since packed or stacked bottles can cause greater variations in temperature between samples. Depending upon the original temperature of the water, there would be different degrees of impact on the bacterial activity in the sample. However at storage temperatures of 4±2°C, most bacteria become much less active. This means that the samples can be stored for longer (generally up to three weeks) before some of these bacteria become active again. Whether the sample has been kept for one day or as long as three weeks then they would all have been reduced to a common level of inactivity (static state). Thus

comparisons cannot be made between samples stored for only one day with those stored for as long as three weeks and then the comparison would have limited value restricted to just those samples.

In setting up the BART testers using these samples it is very important to have allowed the water samples to have returned to room temperature. To do this put the sample bottles out on a bench without stacking them or pushing them together. There needs to be a good flow of air around each bottle to ensure that all of the water samples have come up to room temperature (22±2°C) before beginning the testing. Of course all of the water samples would be impacted by a cooling and then warming cycle which would affect the levels of bacterial activity but hopefully in a relatively common manner. It may be expected that the time lapses would normally have lengthened due to the additional time that the bacteria have now taken to adapt. While reactions may not be affected by the prolonged storage it could be expected that the time lapse (and hence the prediction of the pac/ml) would have lengthened with smaller predicted populations. However these data can be used comparatively for the various samples subjected to the same storage regimen. Generally any storage time of greater than 24hours even over ice limits the value of the data to semi- qualitative and quantitative values. If water samples are involved in one to four days of transport to the laboratory for testing then bacterial activity and populations can be measured but may only be used in a comparative sense with data from other sample sets subjected to the same conditions.

Bottom line is that you can store water samples in a refrigerator for as long as three weeks and you will be able to conduct BART tests for the major bacterial communities. Remember that the bacteria would be affected and some might actually thrive at these low (storage) temperatures

while others would take some time before flourishing. That is the reason for putting a three week upper limit on refrigerated. Also if the water sample is from a very cold source (e.g. (8±4°C) then these bacteria might adapt quickly and dominate even when the testing is done at room temperature.

4.7. BART testing at sea

Ships have two major internal bacteriologically influenced problems with water that can affect ongoing operations. These problems relate to: (1) the potable water supplies for any crews and passengers, and (2) the bilge waters that collect between hull and/or keel plates. BART testers can be used to determine the extent and risks that can be associated with the risks that can be generated from too high a level of bacterial activity. To address these two problems, it is recommended that the following three testers be employed to test the activity of:

- General heterotrophically active bacteria using the HAB-BART tester;
- Sulfate reducing bacteria using the SRB-BART tester; and
- Acid producing bacteria using the APB-BART tester.

While the HAB- tester will detect unacceptably high levels of bacteria in potable waters, the SRB- and APB- testers can both be used to monitor bilge waters for potential corrosion. To conduct each of these tests then 15ml of the sampled water needs to be added to tester following recommended procedures. It is recommended that the more economical laboratory testers be used in racks. These racks need to be

held down to prevent ship movements from affecting the testers. Reading the BART testers is very simple:

- HAB-BART tester the sample solution is blue at the start of the test. If the blue disappears from the bottom up (UP reaction) then aerobic bacteria dominate and there is a lot of oxygen in the sampled water. If the blue disappears from the top down then there is strong likelihood that the bacterial activity could be supporting corrosive reductive events within the water. Here the population activity is directly linked to the delay before a reaction is seen. The longer the time lapse then it may be linked to the smaller the active population of general HAB bacteria. Time lapses are usually measured in days and for potable water supplies the blue color should stay for at least four days (preferably six). If the blue color bleaches in less than two days for a potable water supply then disinfection of the water should be a considered option. Where bilge water is being tested with the HAB-tester it can be expected that larger bacterial populations will be active. The occurrence of a down reaction in the tester could be taken as a warning sign that corrosive processes are under way in the sampled bilge. Generally for bilge waters a time lapse of less than two days may be considered significant particularly with a down (DO) reaction. Refer to the data from the other two BART tester types for clarification of the corrosion risk in the bilges.

- SRB- testers determine the risk of pitting and perforation of the steels. There are two reactions that can occur: (1) blackening around the ball called a BT reaction; and (2) blackening in the conical base of the tester called a BB reaction. BT links to widespread pitting and BB links more to perforation

of the steels. Both of these are warning signs that corrosion could be affecting the safety of the ship.

- APB-BART testers have only one reaction which is a dirty yellow (DY) color that relates to the formation of organic acids that can aid in the pitting of the steels. Time lapses are significant with less than 5 days for an SRB- and 3 days for the APB-BART indicating a significant corrosion risk in the bilges.

BART testers can therefore be used to determine the extent and risks that can be associated with the risks that can be generated from too high a level of bacterial activity. To address specifically potable water supply challenges, it is recommended that the general HAB bacteria test be used employing the HAB- testers. To conduct each of these tests then 15ml of the sampled water needs to be added to tester following recommended procedures. Once set up the testers are left at room temperature and it should be noted that the normal ship motion has been found not to significantly affect the BART testing process. There are two reactions that normally occur. If the blue disappears from the bottom up (UP reaction) then aerobic bacteria dominate and there is a lot of oxygen in the sampled water. If the blue disappears from the top down (DO reaction) then there is strong likelihood that the bacterial activity could be supporting off-odors, colored water and corrosive forms of activities in the water. When you see an UP or DO reaction record that time that this was first observed. The difference between the time the test was set up and the first observation was made gives you a means to predict the population from the time lapse. Here it has been found that there is an inverse but direct correlation between the size of the active bacterial population and the length of the time lapse. The longer the time lapse then it may be linked to the smaller the active

population of general HAB bacteria. Time lapses are usually measured in days and for potable water supplies the blue color should stay for at least four days (preferably six). If the blue color reacts out in less than two days for a potable water supply then disinfection of the water should be a considered option. Where bilge water is being tested with the HAB-BART it can be expected that larger bacterial populations will be active. The occurrence of a down reaction in the tester could be taken as a warning sign that corrosive processes are under way in the sampled bilge. Generally for bilge waters a time lapse of less than two days may be considered significant particularly with a down (DO) reaction. Refer to the data from the other two BART tester types for clarification of the corrosion risk in the bilges (see Chapter 4.8 below).

4.8. Microbiologically influenced corrosion (MIC) and the BART testers

Corrosion is more recognized by its effects than its cause. Effects include leaking tanks and pipes, sudden pressure drops in an industrial complex, increases in the treatment costs, reduced efficiencies in the system, and increased secondary environmental impacts. All of these events are acute problems that require immediate expenditures to achieve control. The chronic causes of corrosion are often forgotten while the acute symptoms are more easily recognized and corrected but often at a high cost. Acute causes of corrosion most commonly are reflected in sudden onsets of perforations leading to leakages and plant system failures. Recognition of these risks is often achieved by building a greater corrosion allowance in the materials but this does not address the cause but merely controls to some extent the effect. Corrosion is defined commonly as the effect of wearing away of a surface (commonly a metal alloy or concretion) as a result of biological or chemical activities. Cause of corrosion is fundamentally two fold.

First the microbes associated with corrosion (i.e. MIC) would need to be present and active. Second the environment should be conducive to the development of the various events that would lead to corrosion. Detection / diagnosis of corrosion can involve three stages that are not necessarily always performed in the same order:

- Determine the presence of active MIC communities or chemical precursors that could lead to corrosion;

- Diagnose the corrosion risk potential based upon the determination of levels of MIC and chemical precursor activity; and

- Evaluate the nature of the corrosion through its form and function. Ideally the order to conduct the survey would be to go from 1 through to 3.

If corrosion has already occurred then it necessary to: determine the effect (3) and then undertake the establishment of cause (1 and 2). In industrial practices it is often this latter route that is followed.

In the determination of the cause of corrosion through a recognized MIC activity, the first step should be to determine whether there is any microbial activity. One simple first step methodology is biochemical and fast involving the assessment of the ATP (adenosine triphosphate) in samples from the site. If there is biological activity then there would be ATP activity (as the prime energy driver) associated with the growing MIC biomass. Once ATP activity is confirmed then one of two MIC bacteria can be identified. There are two cultural methods that can determine the activity of the sulfate reducing bacteria (SRB) and the acid producing bacteria (APB) using the tester system. These BART methods allow corrosion risk to be assessed on the basis of the activity (recorded as

time lapses) and observed reactions. If ATP levels are high and the SRB- and/or APB- data shows very active bacterial communities (positive reactions with short time lapses) then the causative agents can be recognized.

Diagnosis of the corrosion risk in the sampled environment is based firstly on the ATP which is measured in picograms with significant MIC presences being at greater than 100pg/g or pg/ml. For the SRB- and APB- BART test data the critical risk would be generated when the time lapse is less than three days. In the SRB-BART test then a BB reaction would indicate that it would be more difficult to manage because of the more covert (pitting - perforation) nature of these growths. BT reaction is generally more manageable since here the SRB are sited deeply within the biomass and these can be treated more effectively by disruption of that biomass. APB-BART data has one reaction (DY) and this type of MIC is more associated with lateral slow growing biofilms (associated with organic acids generation) that eventually lead to more generalized failures.

Nature of the MIC at site may be examined by looking for pits and perforations, in encrustations, nodules, tubercles, ochres, and various forms of biomass plugging. It is also important to determine whether there are any significant electrical motive forces (e.g. buried power cables) that might be attracting the attention of MIC.

4.9. Chlorine disinfection and potential impact on BART testing

Chlorine disinfection treatments in water commonly use different strengths of bleach as standard treatments for water wells suffering some form of production loss or quality control problems. These symptoms of failure could at least be partly caused by the forms that the biomass takes

within the water environment. Natural growths and activities of bacteria can cause plugging, encrustation, slimes, corrosion, discolored water, smells and can even affect the amount of water being pumped. Within water, the microbes are commonly dominated by various groups of bacteria and chlorine has been found to affect most of these bacterial growths and activities and reduce symptoms. Of the chlorine products it is sodium hypochlorite as a 5.5% solution that is most readily available (as domestic bleach). This product is a very economical way to apply shock chlorination. Symptoms that commonly cause problems for the water users include losing flows, offensive odors (such as rotten eggs), dirty or discolored water, and frequent equipment failures due to corrosion or plugging. BART testers can be used to identify some of the bacteria that are the principal cause of these failures. All testers contain chemical neutralizers that would prevent the chlorine from interfering with the activities and reactions generated by bacteria. If testers do show activities and reactions indicating bacteria are present before treating with chlorine then successful treatment could be confirmed by repeating the tests and finding either much longer time lapses (smaller active populations) and/or shifts in the reaction patterns (different communities). Testers showing reactions can determine the types of active bacteria and these reactions can be used to crudely determine whether how much chlorine treatment has impacted on active bacterial activities. Remember to follow all of the recommended safety procedures (example, safety goggles be worn and that the hands be protected by wearing latex or rubber gloves) when handling chlorine. These procedures may also be application (e.g. gloves and goggles) when setting up testers on chlorinated samples

If the chlorine is effective at reducing bacterial activity then changes may be seen through lengthening time lapses

and changes in the form of reactions seen in the tester. Common effects of chlorine are that the colors are lighter, growths to break apart more readily, and some level of clarity returns to the water in the tester. It should be noted that chlorine (at concentrations of up to 5,000ppm) would be normally neutralized in the tester. Furthermore remember that the positive testers may contain active microorganisms and disposal should follow the standard recommended procedures as described on the Certificate of Analysis that can be found in all BART boxes.

4.10. Relationships between time lapse and predicted bacterial populations, colony forming units (cfu) and predicted active cell (pac) comparison.

BART testers work on the concept that specific bacteria within a given sample would be able to generate activities or reactions in which the time lapse generated reflects the active population of those bacteria. Here less active populations would involve longer the time lapses before activity and reactions that would be generated by. Furthermore it would be only such active bacterial populations within the sample that would generate the time lapse. In predicting the active population of bacteria, replicate testing needs to be undertaken on many water samples of known populations to determine time lapses. These analyses generated statistical relationships between the time lapse and the size of the active population of bacteria in those samples. To achieve these relationships, correlations were made with data from conventional agar spreadplate technologies employing serial dilution to obtain comparable populations as colony forming units using agar plate techniques. Measurements using colony forming units per ml (cfu/ml) have been around for more than a century and this data has been based on the convenience of being

able to count the numbers of distinct bacterial growths (called colonies). The more colonies that are counted then the greater the population estimate of detectable culturable bacteria in that sample. This has become a standard for reporting in bacteriology with colony forming units per ml (cfu/ml) being accepted as the standard term.

Using the agar spreadplate methods has a number of drawbacks which include: (1) the common need to dilute the sample so that the sample being tested contains between 30 and 300 culturable cells which limits scope; (2) the agar surface provides an unfriendly environment for many bacteria to grow on and form a colony with those bacteria that are not culturable being not counted since they did not form colonies; (3) agar plates generates restrictive environments in which the water is bound up under highly oxidative interface; and (4) spreadplates do not offer a variety of environmental sites within which colonies can form. These factors all impart stress that can limit the sensitivity of the agar culture media due to the inability of many bacteria to form colonies and be counted.

BART testers offer a variety of environments within which the bacteria in the undiluted sample can become active. These environments are generated primarily along oxidation-reduction and selective nutrient culture medium diffusion gradients. The water within the tester is basically from the sample and so there is no trauma for the bacteria that would otherwise have been caused by dilution. This means that the test begins immediately the sample is added to the tester and positive detection relates to the time lapse before recognized activities and reactions are observed. These activities or reactions relate to the type of bacteria detected while the time lapse can now be statistically converted to predicted active cells per ml (pac/ml). In generating pac/ml the statistics have been established using pure bacterial cultures, natural samples commonly

employing the agar spreadplate techniques in which the data is generated in cfu/ml. There is therefore a direct link between the cfu/ml in the statistical formulation of the pac/ml using the tester technologies. For this reason it may be taken that pac/ml can be considered equivalent to cfu/ml on the understanding that is some ways the tester offers improved sensitivity and better precision.

4.11. Health of the environment and users of the water

Health can mean one of two things. Firstly there are the health risks to the people directly and indirectly using the water. Secondly there are the health risks to the water system itself (e.g. water well, storage tank, treatment process) itself. Public health addresses the former concern with extensive monitoring programs and so will not be discussed further here. The health of the water itself is another concern. Traditionally the health of water has been addressed in engineering terms relating to production rates and acceptable quality maintenance issues. Newer concepts relating to water has to also consider risks from biomass infestations associated with the water source, collection and delivery can also affect the acceptability of that water. There will almost inevitably be some level of microbiological activity associated with the delivery system that can affect water quality and production. This microbial activity can relate to may have on the product water quality through bioaccumulation of chemicals from the water (filtration effect); releases of microbes and their daughter products into the water (sheering effect); and the direct impact of the growing biomass in flowing water (plugging effect).

These three effects can all contribute to the deterioration in water quality and production and thereby affect the processing of the water for consumption. Additionally the

growing biomass is likely to including reductive zones where corrosion of support equipment is likely to occur (see MIC, Chapter 4.8) which forms another challenge to the health of the water system. What is happening here is that primarily bacteria are concentrating on forming a biomass generally at the oxidation-reduction fronts where oxygen is coming from the oxidative sides and chemicals and nutrients coming in with the water from the reduced side. This biomass focussing initially starts as a natural biological filter improving the quality of the water by bio-accumulating chemicals such as iron or degrading recalcitrant organics. As the biomass grows not only is there commonly a reduction in the flow / specific capacity but also the chemistry of the produced water changes. This decline in water quality is more a result of the biomass beginning to fail to function as a “natural” filter but releases some of the chemicals that had been accumulated. At the same time some of the bacteria active within the biomass will also be impacted by these destabilizations and there would also be periodic releases of bacteria into the produced water. Water quality and production therefore shows periodic spikes in undesirable bacterial numbers and chemical content that then terminates with step-wise increases as the biomass collapses.

Chemical testing of the product water commonly will show increases in the metal content (particularly iron) along with increases in the particulates and total organic carbon. BART testing of the product water will first display more erratic ongoing increases in bacterial activity recognized by shortening time lapses and often changing reaction patterns. Shortening time lapses means the bacteria are getting more active and this may likely to be affecting the “health” of the water supply.

4.12. Zones of Interrogation (ZIP), Microbe hunting using BART testers

We live on the oxidative surface of water-rich planet that has a primarily reductive crust. This means that when groundwater is extracted it moves from a reductive state (in the crust) to an oxidative state on the surface. Biological activity occurs in oxidative state for plants and animals but microbes can sometimes grow in reductive conditions. Here there has been found to be some preference for growing at the interface between oxidative and reductive conditions. In examining environments for different bacterial communities (e.g. ground water extraction wells) it has often found that these communities are actually growing along the gradient that exists between strongly oxidative and reductive conditions. In general bacterial communities will cluster along this gradient in the following order (oxidative to reductive): N-, IRB-, HAB-, SLYM-, SRB-, and DN-. The latter two bacterial communities are not yet recognized with customized BART testers since they occur under very reductive conditions. CH4- communities generate primarily methane from the reduction of fatty acids and carbon dioxide while CR- communities reduce organics to hydrocarbons and elemental carbon. The first six bacterial communities listed are ones that can be monitored using BART testers. In groundwater investigations it has been found that these six communities can be detected by their location and activity in sequences of timed pumped water samples from extraction wells.

Here it is critical to disrupt the biomass first to maximize the sheering of bacteria during after this disruptive phase. For a producing well this may be as simple as turning off the well thus breaking the production cycle and causing the oxidative-reductive interface to shift towards the borehole. Once disruption of the environmental cycle has occurred then pumping the well continuously will cause the sheered

biomass to come out in sequence. For example the early pumped bacteria would have come from close to the well (e.g. IRB-) while later pumped samples would be from further away from the borehole (e.g. SRB- and DN-).

By conducting BART testing on the sequence of pumped samples it is possible to determine where the various bacteria communities are in relation to the borehole. BART-SOFT version 4 allows the data to be entered to allow the relative positions of the bacterial communities. Data entry includes the sampling time into the continuous pumped water cycle, time lapses, and reaction patterns that then allows the generation of the zones of interrogation. Here the borehole is presented as a series of concentric rings and the activity of each bacterial community is shown by color (red – very active; yellow – moderately active; green – present at background levels; white – not detected).

Zones of interrogation (ZIP) refer to the levels of activity determined for each of the sequentially pumped samples. Here the red concentric rings represent the positions around the borehole where that specific community (e.g. IRB-) is active. Clearly a bore hole showing red concentric rings for a given bacterial community activity would be sites where these bacteria are capable of having contributed considerably to the biofouling. If ZIP are repeated after well treatment then the changes in the positions and activities in the borehole would give some indication of whether that treatment had been successful at reducing bacterial activity (e.g. red going to yellow or green would indicate that control of activity had been achieved by the treatment).

4.13. ATP testing for microbial activity

Adenosine triphosphate (ATP) is the principal molecule used for the storage of high energy within phosphate bonds.

ATP is universal in all living cells and performs high energy storage functions. When cells are metabolically more active then the ATP concentration tends to rise. Concentration of ATP is measured in picograms per gram (pg/g). Dormant cells have virtually no ATP while active cells generate concentrations of ATP in relationship to their activity level. Thus testing for ATP as an indicator of bacteriological activity in environmental samples can be achieved in at least a semi-quantitative manner. Methodologies for the detection of ATP have focused on the ability of the enzyme, luciferase, to break down ATP quickly with the generation of light directly from the breakdown of the high energy phosphate bond. The greater the amounts of light generated then the greater the amount of phosphate bonds that were broken down. The source for luciferase enzyme was initially the firefly (*Photinus pyralis*) or the bioluminescent bacteria (*Photobacterium*). This test can be conducted very quickly which has made the ATP test a "gold standard" for the detection of quantifiable biological activity.

Initial research on the potential use of bioluminescent as microbiological activity detection method was proposed in 1968 for use in waters and then in foods by 1970. Since then ATP methodologies has replaced the traditional spreadplate techniques where the bacteriological activity levels in the sample of prime interest. Since that time luciferase testing for bacterial activity has undergone significant improvements to improve precision towards being fully quantitative. ATP assays measure the amount of ATP in the sample commonly as relative light units (RLU, also sometimes called relative luciferase units) which can now be more directly related to the viable active bacterial population. ATP has now also been adopted for the presence/absence detection of bacteria on surfaces and also for the detection of the number of active cells. This approach does not necessarily differentiate the source of the ATP activity beyond prokaryotic (bacterial) and eukaryotic

(higher organisms) cells but lacks precision in the evaluation of specific groups of microorganisms. Thus the ATP test can be used to confirm in a semi-quantitative manner that there is activity within a sample of the consorm. RLU is measured by the amount of light emitted during the interaction between luciferase and ATP in the presence of oxygen. This is summarized in the equation below:

$$Luciferine + ATP + O_2 \underset{Mg^+}{\overset{Luciferase}{\rightarrow}} Luciferine(oxidized) + AMP + CO_2 + Light$$

ATP detection is accomplished using the second generation QGA – Quench Gone test kit that is available from LuminUltra Technologies (440 King St., King Tower, Suite 630, Fredericton, New Brunswick, Canada E3B 5H8, www.luminultra.com). This method uses a luminometer to measure the amount of light produced during the test, along with Luminase (luciferase) solution that should be kept cold in a refrigerator, UltraLute for dilution of the sample, Ultralyse 7 and tubes A, B and C for each test. Calibration of the Luminase is important before starting each set of test since the luciferase will weaken over time. To do this calibration two drops of (100µl) of Luminase are added to 2 drops (100µl) of UltraCheck 1 in a small (12x55mm) assay tube. This is now mixed gently and then immediately inserted into the powered up luminometer and the enter button is pressed. After ten seconds the screen will display a number which the calibration RLU_{CL} value. If the value obtained is less than 5,000RLU then the Luminase is spent and a new calibration would need to be done with a fresh bottle of Luminase. If the number is greater than 5,000 then this should be recorded and used for calculating the ATP.

For liquid samples (or suspensions of solid samples, 5 or 10% dilution recommended) the technique recommended is: (1) For EACH sample, add 1ml of Ultralyse 7 into a 17x100mm extraction tube (TUBE A), 9ml of UltraLute

into a 17x100mm dilution tube (TUBE B), and 2 drops (100µl) of Luminase into a 12x55mm assay tube (TUBE C); (2) Thoroughly mix the sample, and then add 1ml to TUBE A with the Ultralyse. Cap the tube and mix thoroughly; (3) Allow sample to sit for at least 5 minutes to allow solids to settle; (4) Carefully remove 1ml of the supernatant and dispense into TUBE B. Extra caution is required at this point to ensure that any sediment at the bottom of the tube not be disturbed as this will add to an anomalous reading. Cap the dilution tube and mix thoroughly; (5) Transfer 100µl of diluted sample to TUBE C and immediately place assay tube into the luminometer, press Enter, and record RLU_I displayed after 10 seconds; (4) Convert RLU_I to Total ATP_I (pg/ml) using the following formula:

$$\text{Total ATP (pg/ml)} = (RLU_I / RLU_{CI}) \times 20{,}000$$

When calculating the total ATP as pg/ml (if liquid sample) or pg/g (if a solid sample) then the total ATP would have to be corrected for any dilution factors used in the preparation of the sample. Normal range of ATP found in bacteriologically active samples would range from a low of 250 to more than 1,000,000pg/ml or pg/g. Generally total ATP values of less than 2,000 pg would be considered relatively inactive, <200pg virtually inactive, 5,000 to 20,000pg active, and greater than 20,000pg very active. In the event of virtually none (<200pg) and relatively inactive (200 to 2,000pg) of total ATP by the methodology described above then there may a need to enhance the potential for metabolic activity by stimulation through enrichment. This enrichment technique is designed to determine if there is a potential for greater ATP activity if the consormial sample was stimulated.

4.14. Temperature influence on BART testing

Temperatures from which samples are taken can have an impact on the precision in the microbiological cultural test data based on specific predetermined incubation temperatures used. One factor that has to be considered in microbiological testing is the incubation temperature to grow (culture) the microbes. There is some differences based upon where the microbes are active. Microorganisms living in, or around water wells tend to have very stable temperature. This is unlike conditions in soils and surface waters that are subjected to some level of day-night temperature fluctuations. In the one and a half centuries, microbiology has been dominated by the search for microbial pathogens of warm blooded animals that function at temperatures ranging from 35 to 45°C. One unfortunate outcome of this was the idea that all microorganisms would grow best at these temperatures (35 to 45°C). By the 1930s it was realised that many microorganisms had lower optimal (most favourable) growth temperatures and by the 1970s, 28 to 30°C was considered to be the most suitable. There are now four temperature ranges that can be considered for the detection of microorganisms by cultural testing. These are: 12±2°C; 22±2°C; 28±1°C; and 36±1°C. Each of these temperatures is used for different communities of microorganisms that can function in different environments.

Natural incubation temperatures to culture these cold-loving microorganisms can be most effective at 12±2°C with severe trauma setting in on often when the temperature gets up to greater than 16°C. Many of the microorganisms growing under these cold loving conditions can also grow at higher temperatures and these can also be cultured at 22±2°C (room temperature). Microorganisms cultured at this temperature would not be growing at their maximum rate (optimal) but there would be a broader spectrum of microorganisms able

to grow including many of the cold loving and some of the warm loving microbes. This temperature range (22±2°C) has the convenience of being normal room temperatures in most countries and so easy to set up. That is one of the main reasons why the original BART testing was recommended to be done at room temperature. Care should however be taken to ensure that temperature does not fall below 20°C since the range from 16 to 19°C can produce erratic cultural activities and a lower precision in the data generated. For microorganisms growing in the temperate environment where temperatures range from 16 to 34°C, the optimal incubation temperature has been found to be 28±1°C. Using incubation temperatures at higher than 29 or lower than 27°C tends to cause losses in precision. For warm ground waters in tropical climates and mildly geothermal extraction wells, the microorganisms would operate over ranges similar to the warm blooded animals and the most suitable temperature for culturing these microbes would be 36±1°C.

Population counts (achieved by agar spreadplates as colony forming units, cfu/ml), or predicted active cells (achieved by BART testing systems generating time lapses convertible to predicted active cells, pac/ml) are both responsive to the incubation temperature in different ways. For the agar spread plate, the lower incubation temperatures do significantly affect the length of the incubation time to generate colonial growths. Generally the times before counting colonies are: 12±2°C, 21 days; 22±2°C, 10 days; 28±1°C, 7 days; and 36±1°C, 5 days. For colonies to be countable, they first of all have to be able to form a large enough size to be countable diameter (0.2mm to >2mm). Colony counts will generally underestimate the number of microorganisms because of two factors:

- Only a small fraction of the microorganisms are able to grow under the conditions that are generated in the agar; and

- Competition between rival colonies as they form will cause some colonies to be destroyed before becoming countable.

4.15. Impact of salts on bacterial activity

Chemical salts, often dominated by sodium chloride, are a common component in all environments from the oceans to salt flats and caverns. These salts are a dominating factor restricting the types of microbes that can be active. Generally it is sodium chloride that is one of the dominating chemicals in dissolved or saturated natural salts. It is therefore common practise to define the concentration of salts by their equivalence to sodium chloride. In the oceans the salts are commonly referred to as “sea water salts” and gauging the effects of salt is done using natural mixtures of seawater salts. Microbes tend to be more resistant to salt concentrations than most plants and animals which generally will function over extremely limited ranges. Ranges affecting microbial activity, in general, are:

- Sensitive, total salt concentration of less than 200ppm. These microbes are extremely sensitive to salts and are active only when the water is virtually free of salts of any kind (e.g. rainwater, ice melts).

- Normal, 200 to 80,000 ppm (0.05% to 8%). Of the microorganisms it is the bacteria that appear to have the greatest tolerances within the “normal” range. Within this range there are optimal concentrations of salt that have a minimum effect on bacterial activity and outside of that range then the bacteria may become effectively inactive. However little is know of the ability of bacterial consorms (communities) to construct a biomass that effectively controls the admission of salts and, in so doing, controls the potential impact of salts.

- Salt tolerant, 80,000 to 140,000ppm (8 to 14%). Once the environment enters a salt regime of between 8 and 14% then this becomes a major impediment to the activities of all microorganisms except those that are salt tolerant. These salt tolerant communities tend to dominate within zones of the salt gradients.

- Salt Resistant, >140,000ppm (>14%) to saturation. These salt resistant microbes dominate the environments when salt is between 14% and saturated. Their metabolism is likely to be salt dependent which renders laboratory culture much more challenging

Most surface and marine environments are within the "normal" range for bacteria with most tolerance for salt being over the 1,000 to 40,000ppm (0.1% to 4%) range with many bacteria being unable to function in the 4 to 8% gradients. Thus in the literature different salt tolerances are seen for different species but the protective function achieved by a consorm (community) could significantly affect these impacts.

4.16. BART testing cloudy and turbid samples

One challenge in BART testing water samples rises when the water sample is highly turbid. For example when collecting casing samples it could well be that the water is heavily laden with iron debris (red water). Such samples cannot be used at full strength (15ml per tester) since the turbidity would directly impair the recognition of specific reactions (e.g. HAB- would be difficult to determine the start of the UP or DO reactions, SLYM- could not clearly

differentiate the start of particularly CL reactions, IRB- would make it impossible to detect CL, BC and BG reactions, and SRB- would be marred for the recognition particularly of the BB mainly because of sedimented debris). Filtering the sample through a 0.45, 2.0, 8 or even 12 micron porous filter while it would improve clarity would also lead to removal of many of the clusters of bacteria thus improving clarity but at the expense of accuracy. Filtering would therefore not be a very successful technique for improving clarity of very turbid waters for BART testing since there would be erratic removal of bacteria by the filter.

The recommended technique for cloudy or turbid water is to dilute the sample in (preferably sterile) distilled water (do not use deionised water) with 1.5ml of original sample and adding 13.5ml of water. This disperses the turbidity most of the time to allow the evaluation of HAB-, SLYM-, IRB-, and SRB-. If the tenfold dilution does not provide clarity then go a hundredfold dilution (0.15ml sample in 14.85ml water). Correcting the population to observe the dilution then multiply the population by x10 for the 1.5ml diluted sample and by x100 if 0.15ml diluted sample was added. Approximate populations are given on the "Certificate of Analysis" which accompanies every box of testers manufactured. Cloudy waters may be considered to include those waters that are not discolored but have a reflective grayness which makes printed letters when viewed through the sample difficult to read. Turbid samples are more likely to be colored with suspended particulate loadings that prevent the reading of any text through the sample.

In using the dilution technique to clarify the water sample for testing it needs to be remembered that the act of dilution may cause some stress in the indigenous bacterial population that could lengthen time lapses, and the nature of the diluant may also have a negative impact on bacterial activity. Sterile distilled water has been found to be the least

intrusion diluant and so is recommended. Other diluents that have been used are isotonic saline, and phosphate buffers but lack the convenience of sterile distilled water. See Chapter 5.1 and Table 5.1.1 for further details when dealing with turbid and black water samples and soils.

Chapter 5

Sample Preparation

5.1. Preparation of Samples

BART testers can be used for water samples directly by the admission of 15ml directly to the tester. However there are occasions when the sample needs dilution to allow the tester to function properly. These conditions are summarized in Table 5.1.1.

Table 5.1.1, BART analysis of turbid waters, soils and growths

Sample type	Amount	Diluant volume	Method	Diluant	Correction Factor
Turbid waters	1.5ml	13.5ml	A	SDW	x10
Black waters	0.15ml	14.85ml	A	SDW	x100
Sandy loam	1.5g	13.5ml	B	SDW	x10
Loam	0.5g	14.5ml	B	SDW	x20
Clay loam	0.1g	14.9g	C	SDW	x150
Concretion	1.0g	14.0g	B	SDW	X15

Note: turbid waters are those waters too thick or colored to allow clarity sufficient to recognize the testers reactions; black waters are those waters that appear to be black but do not necessarily contain a high particulate content; loams are defined as being predominantly sandy, normal, or a clay loam; concretion defines microbiological growths that are occurring within the structure (e.g. nodule, plug layer,

encrustation, tubercle, and rusticle): methods are defined below for each of the sample types; diluant is sterile distilled water (SDW) but in the event of high salt contents in the sample then SDW can changed to either sterile (by autoclaving) 4% or 12% seawater salt solution in distilled water with solution being selected by sample salt concentration (2 to 8% would use 4%; >8% would use 12%); correction factor is the multiplication of the predicted active cell population generated by BART software assuming that there has been a 15ml liquid sample employed and the correction allows the projection of the population per ml or per gram in the original sample (as pac/ml or pac/g respectively); note that pac can be directly converted to colony forming units (cfu) if required for comparative or regulatory purposes.

There are three methodologies (A, B, C and D) defined in Table 5.1.1 which require a different procedure that the standard defined in the "Certificate of Analysis" which accompanies each box of BART testers. The changes are defined below:

A. This method is specifically for liquid samples that have poor clarity and could not be used at 15ml volume. Here the procedure involving these types of samples would involve unscrewing the cap of the inner tester (placing it on a clean surface without turning it over), and adding the defined volume of sample (e.g. 1.5 or 0.15ml) directly over the ball in the base of tester. It should be noted that this assumes that there are no particles in the sample that are greater then 0.1mm. If there are larger particles than that in the sample then there is a risk of ball jam (where the particles collect around the equator of the ball and prevent it from rising). Here revert to method B. Once the liquid sample has been added successfully with no ball jam evident, then add the SDW to bring the liquid

up to the fill line (13.5 or 14.85ml). The ball will float up as the SDW is added. It should be noted that particularly in the case of black waters there would be a diffusion front formed as the black water moves up into the SDW column in the tester. Do not shake the tester but allow the process of natural diffusion to occur. Note that in the case of HAB- and APB- testers there has to be a modified methodology described below under D.

B. This method can be employed for lighter soils and concretions. Here there is a high probability of particles leading to ball jamming and so the ball has to be removed prior to adding the sample. The sequence for adding the sample is as follows: (1) unscrew inner tester cap and place upside down on clean dry surface; (2) roll the sterile ball out into the sterile inner surfaces of the upside down cap; (3) weight out sample and deliver to the base of the tester using a sterile or flamed spatula, see flaming spatulas in Chapter D below; (4) dispense SDW as the diluant as specified in Table 5.1.1.; (5) lift the cap containing the ball and roll the ball back into the tester; and (6) screw back the inner tester cap. Do not shake or agitate but allow the sample to gradually equilibrate with the diluant.

C. Sticky clays and clay rich loams create different challenges to testing in that the sample can disperse causing clouding and causing ball jams. For these reasons the method employs only 0.1g of sample and the method followed is the same as for B above except that stages (4) and (5) are reversed. Here therefore the ball is returned to the tester before the diluant is added. The reason for this reversal is that the ball being admitted before the diluant allows the diluant to

flow around the ball and mix with the sample. This allows a more even dispersion of clay-based sample.

D. Modified protocols for sterilizing spatulas by flaming and, for HAB- and APB- testers only, reducing the risk of serious of ball jam during set up procedures. Stainless steel spatulas need to be sterilized before use and this can be done by steam sterilizing (autoclaving) within a sterile package; or the spatula can be sterilized by heat immediately before being used by moving in to and out of a Bunsen flame for five seconds. Make sure that that spatula does not get too hot to safely hold by the handle. For the HAB- and APB- testers, the protocols described above call for the tester to remain unshaken (undisturbed) during set up. This means that the application of the chemical (methylene blue for HAB-, and bromocresol purple for APB-) in the cap has to be dissolved and mixed into the body of the tester without shaking. This can be achieved by pippetting 1.0ml of sterile distilled water into the upturned inner tester cap inside the circular flange. The chemical (methylene blue or bromocresol purple) is dried into the cap and takes one minute to dissolve as a blue or purple solution respectively. While the chemical in the cap is dissolving then the tester could be set up as per standards described above except that 1.0ml less of diluant would be added. This would be because that one ml volume would be added with the dissolved chemical in the cap. Once the tester has been prepared (without the cap) then the contents of the cap are poured over the floating ball in the tester and then capped. Do not agitate, shake or otherwise disturb the tester once capped and racked for observation. It will take approximately one hour for the chemical to diffuse into sample liquid column and generate an even blue or purple color.

There is one condition for the HAB- tester under which it is important to dissolve the methylene blue in the cap rather than follow standard protocols. If the sample contains greater than 4% total salts then applying the standard protocol may cause the methylene blue to change to a green color or bleach out. By pre-dissolving of the methylene blue chemical within the cap using sterile distilled water then the chemical enters becomes blue and is not impacted by the salts from the sample.

In the APB- tester there is a need to ensure that the sample is not acidic (pH, <5.5) since this would cause a premature detection of a positive. Where the soil or water sample has a pH of <6.5 then it is recommended that the pH in the sample (if water) be corrected using sterile one normal sodium hydroxide to 7.0 to 7.5. If the sample is solid or semi-solid and is acidic then the sample still would require adjustment to 7.0 to 7.5 and this should be done on the original sample prior beginning the full APB- BART test.

Chapter 6
Disposal of Testers

6.1. Disposal of used BART testers

BART testers, when charged with a sample and incubated, are likely to contain active bacterial populations whether the tester has gone positive by a recognized reaction for the specified bacteria or not. These testers would then need to be disposed of since they are single use disposable test methods and a potential hygiene risk. Disposal may vary with the location of the completed testers. In the laboratory setting, the testers should be placed in a biohazard bag which would then be sealed prior to steam (autoclaving) or gas sterilization. Once sterilized then the testers would not then present direct health risk issues and should be disposed of with the regular laboratory solid wastes. In the event that the testers have been used at too great a distance from a suitable certified microbiology laboratory for sterilization and arrangements cannot be made to get the used testers to a laboratory for disposal then the testers do have the risk potential through potentially containing active bacterial cultures. To eliminate the hygiene risks then the used testers do need to be disinfected or pasteurized prior to final disposal with garbage.

6.2. Disinfection of used BART testers.

Disinfection means the act of killing "germs". It does not mean sterilization where all living microbes would be killed but just those likely to cause infections. This method of disinfection is recommended as a last report and involves using domestic bleach (nominally 5.5% sodium hypochlorite) soaked into paper towel enclosed in a heavy duty Ziploc plastic bag. Below is an example of disinfection but it can be modified to utilize locally available comparable materials: (1) take an 10" x 10.5" (26.8 x 27.3 cms) heavy duty plastic freezer bag that has a double zipper lock that can securely open and close the bag; (2) open the bag and place perforated sheets of 11" wide household paper towel which have been folded along the longer side of the sheet to

make a “v” shape fold; (3) these folded sheets are placed fold side down into the bag; (4) up to 9 field testers or 15 laboratory testers may now be placed in the center of the bag lying on their side (make sure the outer caps have been screwed down tightly onto the outer vials); and (5) once the used testers have been placed within the folded paper then 50ml of household bleach is poured into the bag which is then sealed for disposal. It is recommended that the sealed bag be now left sealed-side up for one hour to allow the bleach to be soaked up by the paper towel. Should there be any leakage from the contained testers then the disinfecting action of the bleach would control any releases.   After one hour the sealed bag can now be disposed with garbage. Note that the normal function in garbage collection involves compressing the garbage that would cause the plastic testers to fracture and leak. There is sufficient active disinfectant in the bleach to assure the disinfection of the contents so that the risks are no greater than for the rest of the domestic garbage.

6.3.Pasteurization of used BART testers.

Heat can be employed to kill the bacteria that have grown in testers. The recommended method involves the use of dedicated 800 to 1,200 watt microwave that would only be used for this purpose. To perform this treatment the initial steps are the same as given above for disinfection using heavy duty microwave safe plastic Ziploc freezer bags. Up to 9 field testers or 15 laboratory testers can be placed wrapped in the household paper towel. These spent testers should be set up right and the caps screwed tightly down. The microwave should be run for 50 seconds for up to 9 field testers or 65 seconds for up to 15 laboratory testers. This amount of heat would be sufficient to pasteurize the contents without distortions in the plastic testers that would allow the contents to leak out. If this does happen then the leaks would be absorbed by the paper towel. After the heat treatment essentially pasteurizing the tester’s contents then the sealed Ziploc bags can be disposed with garbage.

Chapter 7

Special Applications of the BART Testers

7.1 Specialty BART uses, water well diagnostics

From 1990 when the BART testers were first sold the principal market was seen to be in the diagnosis of water well problems caused by bacteria with various forms of biofouling as the costly effect. Biofouling takes on a number of forms in, and around, water wells. Principal concerns relate to corrosion (loss of equipment), plugging (losses in production), and deterioration in the quality of the product water. Biofouling events are side effects of the growth of biomass in, and around, the water well. Not all of these events are bad since the biomass also acts as a biological filter taking out and storing some chemicals (like iron and manganese) while degrading others (such as organics). The net effect is therefore as the biomass initially grows the product water quality can actually improve (e.g. less iron, less organics) but when the biomass gets too big it destabilizes (e.g. releasing iron and more organics). These biomass-related events are cyclic which means that a given sampling may only show the state of the bacteria within that sample and maybe not be relevant to the production and quality status of that well. This means that the value of microbiological investigations of wells involves variability in the data generated. It is only by repeated and sequenced sampling that a truer picture can be gained of the status of the well. Selecting the right BART tester is the subject of 7.2; diagnosis of biofouling water wells in the subject of 8.3; and examining the claims made for the effective treatment of biofouled wells is covered in 8.4.

7.2 Selecting the BART testers for water well diagnostics

BART field testers come in a format that makes them easy to use in conditions away from a laboratory. The major difference from the laboratory version is that there is a second tester (bottle) that provides additional stability and protection to the tester when it is being transported and used in the field. There are occasions when there is a need to take a water sample that would then be used to fill the inner testers while out in the field. With the field testers there is the potential to use the outer tester (bottle) as the means of taking the water sample for use in the testers. The inside contents of the field tester are sterile and so therefore, when removed, the outer vial can be used to collect the water sample. To do this use the following procedure: (1) unscrew and remove the outer cap, remove the inner tester and place on a clean dry surface, and lay the outer cap down on a clean surface without turning it over; (2) screw the outer cap back onto the outer vial and it is now ready to be used for collecting the water sample; (3) when collecting the water sample then remove the outer cap again and place on a clean surface; (4) add the water sample to the outer vial but do not fill beyond the fill line beneath the threads, this line denotes that 65ml of water has been added; (5) put the outer cap back on to the outer vial and screw down. A 65ml water sample has now been taken which would be enough to charge four inner testers. It should be remembered that the water sample only remains valid if it has not been contaminated during collection. Therefore do not charge the outer vial in an environment that is dust laden and always handle the outer vial from the outside to avoid contaminating the inside of the sampling bottle. If sterile latex gloves are available then it is advantageous to handle the outer vial wearing the gloves to further reduce the risk of contamination. There are no chemicals added to the outer

vial and so any chemicals present in the water (for example, chlorine) would not be neutralised while in the outer container.

One of the major challenges for the determination of biofouling risk and effective management is that the prime fact that different bacteria growing at different locations around the bore hole. Of these bacterial communities it is often the iron related bacteria (IRB) that are the closest to the bore hole. They have the oxidative ability to accumulate ferric forms of iron inside the biomass or around the slime tubes that they make or push it out of the cells as ribbons. They like a lot of oxygen and are largely responsible for the development of rust-like growths. Using IRB- testers it is possible to detect these IRB by the type of reaction seen. Brown ring (BR) means a reaction specific to the (aerobic) slime forming IRB. If there is a brown clouding (BC) then that means a whole collection of different IRB are active. Normally the first reaction observed is either clouding (CL) which means an oxidative condition or foam (FO) which indicates that the sample was from a more reductive environment.

Just outside of the IRB are the general heterotrophically active bacteria (HAB) that are the "filter feeders" or "organic busters". That means that they feed within the natural biomass filters that build around wells and they have two very important competitive edges: (1) efficiently break down many organics particularly in the presence of oxygen; and (2) also adapt relatively easily to oxygen rich and oxygen deficient conditions. These are the main workers in the natural filters formed by the biomass. Using a HAB-tester will detect these bacteria by one of two types of reaction. These are the UP and DO reactions where UP means aerobic, oxidative; and DO means anaerobic, reductive. In oxidative conditions the UP reaction dominates while in reductive conditions the DO reaction dominates.

Thus sequential sampling of a well can locate the oxidative-reductive interface (redox front) where most of the biomass is active.

Further away in the more reductive conditions around the bore hole the bacteria becomes dominated by the sulfate reducing bacteria (SRB) that generate hydrogen sulfide from sulfates and cause odor and blackened waters. They are driven by the amount of organic acids being generated in the biomass and so are closely linked. There are two reactions (black bottom, BB and black top, BT). BB signifies communities that are more covert and prefer more reductive conditions that are often difficult to treat. BT occurs when the SRB- are growing inside aerobic communities and are both more active and easier to control.

Beyond the SRB in the very reductive regions surrounding the bore hole there are the CH4- (methane producing) communities which generate methane (natural) gas as a major product. When there is a major activity in this community then the methane gas can escape into the bore hole and represent a problem to the well user (well might flare occasionally, head space over the well's water column might become combustible).

Normally the IRB-, HAB- and SRB- BART testers can be include determining the activity of the biomass and the possible location of the redox front. To do this the zone of interrogation projection (see Chapter 4.12 for more information) can be used.

7.3. Diagnosing water wells

When water wells suffer from significant production losses it becomes necessary to treat the wells to get the flow back (preferably sooner rather than later!). Very often specific

capacity readings are taken before treatment and then again afterwards. The success of a treatment is based on the percentage increase in the specific capacity. For example a well that had an initial specific capacity of 5gpm/ft and had a post treatment capacity of 15gpm/ft could be considered to have undergone a 200% improvement in specific capacity. While that may sound impressive the only valid comparison should be with the original specific capacity taken when the well was first developed. If the original specific capacity was 50gpm/ft then taking this as 100% would mean that the treatment only recovered the well from 10% to 30% (5pgm/ft to 15gpm/ft for a well originally developed at 50gpm/ft) which would then be an improvement of only 20% towards the original specific capacity! Always use the original developed specific capacity as the benchmark and not the specific capacity of the fouled well.

In calling for a preventative maintenance or radical regenerative treatment on a well it important to judge success on the basis of the original specific capacity of the newly developed well and theoretically no improvement can be greater than 100%. Many well treatment companies tend to favor the use of the pre-treatment specific capacity to determine effective gains. This is because much greater percentile claims can be made that is not restricted to the 0 to 100% range. Caution should be observed when any claim that exceeds 100% and care must be taken to ensure that the original specific capacity of the newly developed well is being used in the development of any such claims.

Preventative maintenance should only be applied to water wells that have not lost more than 15% of the original specific capacity. Under these circumstances it would be reasonable to consider 10 to 15% improvements after preventative maintenance as being very acceptable. For radical regenerative treatments applied to wells that have lost between 10 to 40% of original specific capacity.

Effective radical regenerative treatments should cause improvements commonly in the 20 to 40% range returning the well to within 10% of its original specific capacity. Generally water wells that lost more than 40% of their original specific capacity cannot be effectively regenerated and improvements of 30 to 40% towards the original may be viewed as successful treatments.

Caution should be taken with any claims that exist 100% because this would mean the claimant may be exaggerating the effectiveness of the well treatment. Given that the original specific capacity was set as 100% then any treatment claimed as percentage improvements in the well and should never exceed 100%.

7.4. Water well treatment claims and reality

The golden rule is "no one size fits all" means that all treatments need to be customised to the water well scheduled for attention and possible treatment. Beware of sales persons who claim that their treatment method will either: (1) cause production after treatment to exceed 100%; (2) be applicable to all wells equally and regenerative treatments will return wells to full production; or (3) this treatment will be the only one that the well will ever need to be given. All of these claims are warning signs that should trigger the "red flag" and should be avoided. Reality is that every well should be treated as a separate challenge and there should be some attempt to customise each well to address differences between them that are observed.

Treatment of a well can be chemical or physical and vary to include a combination of the various treatments when blended. There has never been the successful development of biological treatments that has been shown to be effective in the long term. Traditional chemical treatments can

include a single compound or a blend. Single chemical treatments for water wells have tended to be replaced with blends of chemicals usually used together and commonly including some form of biocide, pH modifier and a detergent/dispersant to kill the biomass, destroy the plugs and clogs, and clean off the surfaces respectively. In general the new blended approach involves phased treatments summarised as shock, disrupt and disperse sequences. At the end of the treatment it is common for the well environment to contain a lot of dispersed biomass that has to be removed. It needs to be remembered that this biomass has grown acting as a filter degrading some organics but accumulating many chemicals. These bioaccumulated chemicals will now be released by dispersion and enter the water. This would mean that a successful treatment of a well could include treating the dispersing biomass as hazardous waste depending upon the bioaccumulates found to be present.

Some treatments involve modifications to applied temperature of the treatment by the addition of heat (to raise the temperature) and coolants (to lower the temperature). Generally raising the temperature during treatment causes faster chemical reactions and greater impacts on the biomass. The blended chemical heat treatment (BCHT) uses this approach. More recently in an effort to be "greener" treatments have been used that are purely physical in nature. Here physical forces are pulsed through the well environment in a manner that is disruptive to the biomass causing collapsing along with dispersion.

Many traditional treatment methods employ chemicals in which phosphorus is employed as phosphate, phosphoric acid or polyphosphates. These could cause stimulation of the post-treatment biomass growth and it is recommended that it should be demonstrated that all of the treatment phosphorus has been effectively removed with the dispersed biomass from the treated well. Failure to do this means that

the residual phosphorus left down hole will stimulate the growth of the biomass that will inevitably form after treatment. This would trigger a heavier biomass generation (on the additional phosphorus entrapped during treatment) to cause very active post-treatment biofouling of the treated well. It would be important to restrict any well treatments to those that do not contain phosphorus as an active ingredient.

Chapter 8

Tester Function

8.1. How the BART tester functions

BART stands for bacteriological activity reaction test which is an apparatus for conducting a single cultural test for the presence of selected bacterial communities (consorms). This apparatus addresses the fundamental mechanisms governing the effectiveness of the tester. While the test employs a total liquid optimized volume of 15ml of liquid sample, the tester has been critically engineered (see Chapter 2 for physical aspects and Chapter 3 for cultural aspects) to allow the culture and recognition of only the selected bacterial consorms registered by their activities and reactions within the tester. In engineering the tester attention was paid particularly to the environments that were created when the tester was charged with a sample (4.2).

8.2. Environments created by the BART testers

Perhaps the most unique feature of the BART tester is that it generates a range of environments ranging from very oxidative around the ball to reductive in the base; to high in selective chemical nutrients rising from the pellet in the base of the tester to background levels around the floating ball. Once the tester is charged with the water then a dynamic process occurs in which multiple environments are created from nutrient rich reductive types in the base to nutrient poor oxidative types at the top around the ball. Essentially, as the selective nutrient pellet dissolves, then the diffusion gradient moves up carrying reductive conditions with it. This diffusion front carries with it the oxidative-reductive

interface where frequently the bacterial activities become concentrated and, in consequence, early activities and reactions are sometimes seen.

For the bacteria in the 15ml sample charged into the BART tester there are a number of interactions that can occur. First there is a rapid reduction in the dissolved oxygen levels in the base of the tester as the bacteria are stimulated by the diffusing nutrient front and utilize the oxygen faster than it can diffuse down the tester. The reductive zone now forms once the oxygen in the base of the tester is consumed. As the aerobic bacteria respire above the reduction zone at a rate faster than the oxygen can diffuse down from around the ball then the reduction front moves up. Thus there is a dynamic in the changing of the environments within the tester based upon the amount of bacterial activity consuming the oxygen, the manner in which chemical diffuse out from the basal pellet generating reductive conditions when the oxygen is spent, and the interaction between the physico-chemical nature of the sample and the chemistry of the diffusing nutrient pellet. Essentially environments in the tester were constantly changing if there were active bacteria reacting with each other and the local conditions within the tester. Each type of tester therefore reflects in unique manners that were expressed (as reactions and activities) within the tester. It should be noted that the term "environment" used here is a relative term and does not exclude the impacts of the environmental conditions outside the tester.

Fundamentally the basic ideas involved in the patented BART concept generating a distinctive environment include the following (see Chapters 2 and 3 for more details include the following:

- Enhancement of the BART tester environment is achieved by the addition of a dried specific culture

medium that allows the BART tester to become supportive for the targeted consorm of microorganisms of interest that may be in the sample under examination.

- Selective culture of any bacterial consorm commonly happens in a staggered manner as the natural shifting of the environment within the tester occurs along with the products of cultural activity causes a gradual change in the dominant bacteria active within the consorm. This would create conditions where there is a bacterial mutuality which supplants competition between the members of the consorm.
- Expression of the tester through activities and reactions would therefore be the direct result of the presence of a suitable active bacterial consorm that had not been suppressed within the sample by any restrictive factors present within the sample. This expression would be a reflection of the cascading ability of the various bacterial members within the consorm to become active, create activities and reactions during testing. The device system therefore involves a dynamic state in which mutualism precedes restriction as various members of the consorm rise to a dominant state which would also involve the suppression of other members of the consorm. In simple terms there is an ongoing "war" between the various bacterial members of the consorm for "growing space" within the tester.
- End points in the BART tester are represented by detectable activities or reactions that occur after a period of time and are recognized as being significant (see Chapter 3) to the positive detection of the consorm that is being investigated. It is recognized that this end point would be influenced by a level of activity and reactions between the bacteria within the consorm. Time lapse, when generated, would therefore represent the mutuality and antagonistic interactions between the active bacteria in the consorm during the preceding incubation of the tester.

- Essentially the BART tester provides an ability to detect the bacterial activity level of the consorm present in the sample after being incubated. It can be expected that bacteria will be moving through the phases of nutrient diffusion upwards (as the medium in the floor of the tester dissolves and rises), and interacting with the physical and chemical nature of diffusing matrices including oxygen moving downwards in the sample column. This would be followed by the generation of limitations (e.g., depletion of nutrients and oxygen and the build up of culturally restrictive end products).
- Precision and interpretation is based upon the time lapse to a recognized state within the tester environment (e.g., going reductive, shifting to a low pH, generation of specific colors or structures within that environment. This interpretation represents the time delay (lapse) over which a complex of microbial cells achieved a given and recognizable state. The time lapse therefore does not directly interpret into a number of cells unless limited pure culture studies are undertaken. It is proposed therefore to consider that the time lapse reflects the status of the consorm under investigation with two proposed states:

ACTIVE is a condition in which the bacterial consorm immediately becomes active in the tester.

STRESSED is a condition in which the time lapse becomes delayed to such an extent that the consorm had to pass through induction (adaptation) before it could become active.

From the investigations to-date it would appear that a time lapse of 2 days, 48 hours or 172,800 seconds would appear to commonly be the interface between an active consorm (<48 hours) and a stressed consorm (>48 hours). At this time correlations between the time lapse and standard microbiological tests are likely to be compromised by the broad spectrum of activities.

Chapter 9

BART Tester Parameter Codes

9.1 BART tester parameter codes (pcodes)

BART testers that are in commercial production do have a parameter code (or pcode) abbreviated to a descriptive short name of 20 characters maximum. Each pcode includes a short description of the tester as a defined substrate technology (DST). Each tester employs a vertically diffusing selective nutritional chemistry that restricts the culture of bacteria communities present in the sample to only those reacting in a recognizable manner to that elevating front. Additionally the tester does generate a reductive-oxidative interface that commonly also rises during the testing period when bacterial activity does occur. All of the testers involve defined substrate technologies designed to examine activities within specific groups of bacteria within the sample being investigated.

Product name: **IRB- BART**

Pcode: **iron biotester**

DST: Tester employs a modified Winogradski ferric-iron culture medium that selectively triggers the growth of both iron oxidizing and iron reducing bacteria within an environment that includes (base to top) reductive to oxidative gradient; and a selective nutrient front that

diffuses from the base to the top of the water column in the tester.

Product name: SRB- BART
Pcode: sulfide biotester

DST: Tester employs a modified Postgate culture medium that allows the growth of hydrogen sulfide generating bacteria. This happens under reductive conditions and so the tester employs a floating anoxic block to reduce oxygen entry into the culturing sample. There are two origins for the generated hydrogen sulfide: (1) sulfates from the selective medium that are reduced as a black base, BB, reaction; and (2) sulfur containing proteins primarily from the sample that are degraded reductively with the release of hydrogen sulfide usually within the biomass growing around the ball as a black top, BT, reaction.

Product name: SLYM- BART
Pcode: slime biotester

DST: This tester employs a rich proteinaceous medium that stimulates the formation of water-bonding polymers that interconnect the cells as a slime-matrix. In the tester this slime growth commonly causes the culturing sample to go cloudy, CL, very quickly often accompanied by gels, thread-like growths, and the formation of foam bubbles that commonly collect around the BART ball as a ring, FO.

Product name: HAB- BART
Pcode: bacterial biotester

DST: In this general bacterial tester a rich selective culture medium containing proteose and peptone-tryptone is employed to stimulate the heterotrophic bacterial growth. To

determine whether the bacteria are aerobic or anaerobic then the reduction-oxidation potential indictor, methylene blue, is used. This causes the color to shift from blue (oxidative, aerobic) to clear (reductive, anaerobic).

Product name: **APB- BART**
Pcode: acidogenic biotester

DST: Fermentative bacteria function anaerobically (reductively) producing fatty acid daughter products. These cause the pH to fall into the acidic range with increasing risks of acidulolytic corrosion. The selective medium contains a mixture of tryptone, peptone, and glycerol to trigger the generation of fatty acids.

Product name: **N- BART**
Pcode: nitrate biotester

DST: This biotester employs a selective culture was based on ammonium sulfate mineral salts and the reaction cap detected the presence of nitrite. When the ammonium is oxidised to nitrate by nitrification by the nitrifiers then nitrates and nitrites appear as daughter products. Nitrate tends to be very transient but nitrite is more persistent and so the test for positive activity is the presence of nitrite. This tester contains three BART balls and is laid on itself to increase surface areas and potential oxidative activity.

Product name: **DN- BART**
Pcode: nitrite biotester

DST: To encourage reductive denitrification by bacteria the selective culture medium contains peptone and nitrate along with important macro-nutrients. Samples containing active

denitrifiers generate nitrogen gases which become entrapped in biofilms as bubbles. These bubbles rise to the ball where a foam ring is formed that commonly lasts one to three days.

Product name: **FLOR- BART**
Pcode: glow biotester

DST: This biotester employs a rich proteinaceous medium that stimulates the growth of pseudomonad bacteria in the oxidative regions around, and immediately below, the ball. Within this zone some pseudomonad bacteria generate fluorescent pigments that glow in natural or artificial UV light and indicate these species are present and active. The recommended UV light is the longer wavelength within the range of 315 to 400nm.

Product name: **ALGE- BART**
Pcode: microalgae biotester

DST: Microalgae are primarily the single celled photosynthesising microorganisms that function in oxidative waters exposed to sunlight. This biotester employs modified Bold's medium which does not contain significant organics, but does contain the basic nutrients for plant growth (nitrogen, phosphorus, potassium, sulfur etc.). Carbon is presented in the tester as bicarbonates (pH, 8.2) to encourage the micro-algae to utilize this form of inorganic carbon. Micro-environments are created within porous cellulosic and plastic weaves to allow localised growths of specific algae while the biotester is laid on its side and illuminated.

Chapter 10

BART Conversion Tables

10.1. Conversion Tables

BART testers have been manufactured in Regina, Canada since 1990 and have undergone a series of improvements that are reflected in the" Standard *Methods for the Environmental Application of BART testers in Investigations of Microbiological Activities"*. Major changes have involved the recognition of the types of reactions and activities that occurred within the various types of BART testers. These have at least been in part the result of BART users who had difficulty with the declaration of activities and reactions within the tester. Initially a numeric system was applied to differentiate these events (Practical Manual of Groundwater Microbiology, first edition, D. Roy Cullimore published by CRC Press, 1993). It became realised between 1990 and 1997 that the use of a number designation was causing confusion and this was changed to a two letter descriptor of the reaction or activity. These new reaction codes were published in "*Microbiology of Well Biofouling*" by Roy Cullimore published by CRC Press, 1999. No conversion tables were made available at that time and some BART users are still employing the original numeric code system and have not switched to the two-letter code. The following tables allow the conversion of numeric to two-letter codes for the reactions and activities within the testers. They are listed below in order: (Table 10.1., IRB-BART; Table 10.2., SLYM- BART; Table 10.3., SRB-BART; Table 10.4., FLOR- BART; Table 10.5., formerly TAB- and now HAB- BART). The other BART testers

were introduced after 1993 and used only the two-letter reaction coding system only and this is addressed in Cullimore (1999).

Table 10.1., Conversion table for reaction numbers to two letter codes for IRB- BART;

1993 reaction number	1999 two-letter code	Comment
1	BG	A
2	BR	B
3	BG	C
4	BC	D
5	FO	E
6	BR	F
7	RC	G
8	GC	H
9	GC	I
10	BL	J
13	ND	K

Comments: A, reaction 1 was a "brown basal swirl" but it was found to stabilize into a brown gel and so was given BG coding; B, reaction 2 was brown ring over a clear solution that was redesignated as brown ring, BR and references to the clarity of the solution was not considered significant for the BR reaction (see also reactions 6 and 7); C, reaction 3 was "brown ring and gel" which has been simplified to basal gel (BG) which may be either ferric-brown or dark green and has been frequently associated with ochres; D, reaction 4 "brown cloudy" now becomes the BC reaction with no change in definition; E, reaction 5 "gas bubbles" has been clarified to simply a foam ring (FO) which occurs only when the gas bubbles have risen to form a foam ring at least 75% of the way round the floating BART ball; F, reaction 6

"brown ring over yellow solution has been reclassified as a brown ring (BR); G, reaction 7 "brown ring red" has been reclassified as red cloudy (RC) with the brown ring (BR) being given separate code status; H, reaction 8 "green cloudy" remains as GC regardless of turbidity in the culturing solution; I, reaction 9 reverts to a GC reaction code since cloudiness (turbidity) is no longer recognized as a prime factor; J, reaction 10 defined as "black deposits" has been redefined as black liquid (BL reaction code); K, reaction 13 is an undescribed negative reaction in which none of the recognized reactions for the IRB- tester have occurred.

Table 10.2., Conversion table for reaction numbers to two letter codes for SLYM- BART

1993 reaction number	1999 two-letter code	Comment
1	BG	A
2	CP	B
3	SR	C
4	BG	D
5	CL	E
5	CL-PB or CL-GY	F
6	BL	G
13	FO	H

Comments: A, reaction 1 is slimy basal swirl modified to a basal gel (BG) since the gel rapidly stabilises when grown; B, reaction 2 is "plates, rings and clouds" now refined to cloudy layered plates (CP) which occur during early phases of growth as either fluffy clouds or thin lateral plates which float generally up the tester to the ball; C, reaction 3 is

"slimy ring" which becomes SR and is normally white or beige but can occasionally be other colors (e.g. yellow, violet, orange, red and brown); D, reaction 4 "slimy ring and gel" now only refers to a dense slimy gel (DS) with the slime ring being recognized as a SR; commonly the DS forms within the lower half of the tester's water column; E, reaction 5 is "white cloudy" which has been found to be a dominant reaction and so is retained as CL (cloudy) reaction; F, reaction 5 also has U.V. fluorescent pale-blue or greenish yellow color that indicate the special nature of these reactions as CL-PB or CL-GY depending upon the dominant color; G, reaction 6 relates to "black deposits" which has been redefined as black liquid (BL) and is caused by either reduced carbonaceous daughter products or possibly iron carbonates; H is reaction 13 or "fuzzy ring" in which there was evidence that fungi could be growing slowly around the ball to form a mycelium but this has not been supported by recent investigations and the reaction has been reassigned to the generation of a foam ring (FO) around the BART ball.

Table 10.3.,
Conversion table for reaction numbers to two letter codes for SRB- BART

1993 reaction number	1999 two-letter code	Comment
1	BB	A
2	BT	B
3	BA*	C
X	CL**	D

Comment: A, reaction 1 is a "black deposit" which is now defined as black base (BB) in which the base of the tester goes jet black along with the bottom 2 to 4mm of the sides; B, reaction "black ring" has been reclassified as black top

(BT) since the sulfide is generated in the biomass growing around the BART ball as small jet black specks that coalesce into a jet black band; C is reaction 3 which is described as "black ring and deposit" and has been described as black all (BA) but this reaction (*) supercedes BB or BT and so is a secondary reaction (in the event of the tester being BA when observed then the first reaction defaults to BB; D, reaction X refers to "partial or complete clouding , no sign of any black deposits" is a term that effectively indicates that no SPB (sulfide producing bacteria) and yet there was considerable bacterial activity (hence clouding, CL**), it should be noted that CL designated as any form of growth or activity not involving the generation of jet black deposits and is a negative for SRB (**).

Table 10.4., Conversion table for reaction numbers to two letter codes for FLOR- BART

1993 reaction number	1999 two-letter code	Comment
1	CL*	A
2	CL-GY	B
2	CL-PB	C
3	**	D

Comment: A, reaction 1 was defined as "cloudy" and is now classified as CL but the asterisk (*) means that this is not a positive detection of either of the groups of fluorescent pseudomonads; B reaction 2 to the left of figure 47, p.310 (Cullimore 1993) relates to "green-yellow fluorescence" and has been reclassified as CL-GY; C, reaction 2 to the right of figure 47, p.310 (Cullimore 1993) relates to "pale blue fluorescence" and has been reclassified as CL-PB; D reaction defined as "clear" is not recognized as a reaction

other than the culturing fluids clear following fluorescence and this should be considered a negative (**) unless preceded by fluorescence.

Table 10.5.

Conversion table for reaction numbers to two letter codes for formerly TAB- and now HAB- BART).

1993 reaction number	1999 two-letter code	Comment
Bleach up	UP	A
Delayed bleach down	DO	B

Comment: A, in the first edition Cullimore (1993) did not clarify the different reaction types in the (then TAB- BART but the one described in the text related to an UP reaction and this is now the standard for aerobic heterotrophically active bacteria; B, no reaction was described in 1993 for a descending (DO) reaction in which the bleaching (reduction) usually began just under the BART ball sometimes with the BART ball turning from pale blue to white, this is now the DO reaction which is common for facultative anaerobes and anaerobes growing under reductive conditions.

Chapter 11

Risk Analysis using BART testers

11.1 Introduction

Risk, whether it relates to health, the environment or an engineered process; is commonly presented in numeric terms. Many people observe the level of risk simply by a number generated by some standard method. In the development of the various BART tester protocols it has been expedient not to include specific numbers (of populations) as being indicative of risk. Partly this is the results of trying to determine what the risk is to (human health, health of the well, efficiency of the system, or perhaps the health of the microorganisms functioning within the biomass of concern?). It is perhaps because the testers are used for a variety of functions in different environments that the traditional role for the testers is to use the data comparatively between each sampling site. In these cases deterioration would normally be seen as increases in populations while effective treatment would show decreases in population. Shifts in the dominant microorganisms would be seen in changes in the reaction patterns observed including the sequence in which they occur. This risk analysis therefore is divided for each biotester into risk to human health, risk to sustainability of the water source, and then general environmental risks relating to the bacterial activity detected. Calculation of corrosion risk (CR), plugging risk (PR) and health risk (HR) using the BART-SOFT version 6 software is addressed in Chapter 13.

Each BART tester has the risk defined separately using both the product name and the parameter code (pcode). Risk ranges are provided based upon the population count (room temperature, 22±2°C) for human health, sustainability of the water source, and potential environmental consequences posed by the risks. Risk assessment is based on the potential for the particular population to be representative of normal background that might be expected in the sample, problematic populations that are sufficiently large to offer a potential problem, and severe where the population detected indicates that a severe threat to the functionality of the environment may exist. Therefore for each BART (bio)-tester the generated time lapse (via predicted population) and reactions can be fitted into a range from normal background, to potentially problematic, and then to severe. In the latter event of severity then the population detected would already be displaying consequences resulting from the populations. Risk analysis is first generated in a single table that defines critical populations (as predicted active cells per ml, pac/ml) that can be taken as equivalent to colony forming units per ml (cfu/ml) using the classical agar methodologies.

In addition to a generalised risk analysis based upon population there is an additional risk analysis based upon the reaction code(s) that were generated during the test. These reaction codes (multiple codes are formed into a chronologically sequenced reaction pattern signature, RPS) are now evaluated as affecting the risk analysis. This analysis is performed for each tester type and is included in the risk analysis interpretation for each tester type.

11.2 Product name: IRB- BART
Pcode: iron biotester

In the iron biotester (IRB-) the objective is to detect the broadest possible range of bacteria that can interact with

iron causing ferric accumulation (oxidative) or ferrous dispersion (reductive). In this test there are a broad range of bacteria functioning under various ORP conditions. Risk to human health relates particularly to the enteric and pseudomonad bacteria which often form integral parts of the biomass. For the sustainability of the engineered system (e.g. water well, cooling tower, heat exchanger) then the risk relates to plugging of the system with biomass or corrosion. Risk analysis is shown in Table 11.2.1 and gives the general risk pattern associated with all observed recognized reactions and then specifically the risks associated with particular reactions.

Table 11.2.1, Risk Analysis for the Investigations of the IRB tester

Reaction	Background	Problematic	Severe
All (a)	50 500	10,000	100,000
BC BR (b)	10 200	5,000	20,000
GC RC (c)	1 100	500	10,000
BL (d)	0 10	100	1,000
BG (e)	10 100	500	5,000

Notes: (a) All reactions refers to the principally the CL and FO reactions that commonly trigger the start of the iron biotester; (b) brown clouded and brown ring reactions relate more specifically to the activities of oxidative (aerobic) iron related bacteria that would increase the risk of iron related plugging; (c) both green and red clouded reactions relate to potential health risk bacteria such as enteric and pseudomonad groups and these increase the risk to health; (d) black liquid is a terminal reaction that may be triggered by either enteric and organic reducing bacteria and (e) would signal either severe health risk or extremely reductive

environments which could impact the water quality severely and affect production.

Environmental risks can be associated with many factors ranging from degeneration of the surface environment (e.g. reducing water quality, obnoxious slimes and seeps) to the natural flora, fauna and microflora. One of the major challenges of the iron biotester (IRB-) is that there are eight recognized phase two, three and four reactions (see Table 3.1.3) each of which can generate both a population prediction (as the first observed reaction) and can also form a part of the reaction pattern signature. In assessing the IRB-risk then there are two aspects that should be considered: (1) population size; and (2) reaction patterns reported. In the rest of the chapter this approach will be employed for each risk assessment.

11.3 Product name: SRB- BART Pcode: sulfide biotester

Traditionally hydrogen sulfide has been linked to the reduction of sulfates in reductive environments. Relatively little attention has been paid to the other principal source of hydrogen sulfide that is the sulfur amino acids that will, in a reductive environment, not only release ammonium as a daughter product but also hydrogen sulfide. Thus there are two sources for the detection of hydrogen sulfide which relate to the source of sulfur utilised by the bacteria these two reactions have a different risk analysis and this is shown in Table 11.3.1. Risk relate primarily to the generation of hydrogen sulfide that can impact water quality, production as well as corrosion. Here the BT reaction would indicate the risk may involve all three parameters with the generating biomass causing blackening in waters, rotten egg odors,

fouling of the natural conduits or pipes carrying the water, and also corrosion commonly electrolytic and generally involve very distinctive black biomass growing in the affective environment that may be reductive or oxidative. It is common for the BT reaction to involve a complex bacterial consorm in which sulfide generators are but one small part usually within the reductive regions of the biomass. By contrast the BB reaction relates to covert sulfate reducing bacteria that commonly grow deeper in the reductive zones (commonly with ORP values from -10 to -150mv). Risk analysis for the BB therefore relates more directly to corrosion risks (generated by the covert biomass dominated by sulfate reducers) rather than the more general flow and quality issues. It is for this reason that there is primary separation of the risk into the BT and BB groups. However when observations of the biotesters are daily then it could be that both the BB and BT reactions may occur in which case this is called a BA (black all) reaction. When this occurs risk interpretation defaults to the BB type of reaction.

Table 11.3.1, Risk Analysis for the Investigations of the SRB

Reaction	Background		Problematic	Severe
BT (a)	50	500	10,000	100,000
BB (b)	1	10	1,000	5,000
BA (c)	20	100	500	10,000

Note: (a) is the risks associated with the black top, BT reaction and normally this would be associated with the degradation of protein-rich organics; and (b) these risks relate the reduction of inorganic sulfates with the releases of hydrogen sulfide.

11.4. Product name: SLYM- BART
Pcode: slime biotester

Slime forming bacteria is the name given to those bacteria that are active within environments and bind water with extracellular polymeric substances. This bulking of the biomass with the bound water can exceed 95% of the total weight. Generally, slime forming bacteria grow under oxidative conditions as floating particulates or attached biofilms; or grow at the oxidative-reductive interface. Heat exchangers, cooling towers and filters are frequent sites where these bacteria grow usually recognized by the abundant amount of slimes attached to surfaces, as floating bio-colloidal particles, and as slime threads forming slime webs through the water. Primary risks relate to the engineered efficiency of the impacted system resulting from reduced energy or water flows through the impact site. Secondarily the impacted water may take on a cloudy appearance and may even show slimy web threads or floating slime particles that make the product water less acceptable. Primarily the slime biotester is a monitoring system for the health of an engineered system to assure that efficiency is not impaired by the slime forming bacterial biomass. Table 11.4.1 gives the risk analysis on the basis of all reactions and then supplementary risks if a dense slime (DS) or black liquid (BL) reaction are observed. These two reactions change the risk analysis. For the DS reaction there is a probability that there is a biomass impacting the water in a manner that is affecting flows (due to plugging) and quality (primarily linked to clouding of the water). Because of the limited location of the DS biomass activities, the effects can be more serious than the numbers would suggest. For the BL reaction which is generally a terminating reaction for the testing then the biomass would have become impacted by very reductive conditions that can severely impact water quality and may also pose a health risk.

Table 11.4.1, Risk Analysis for the Investigations of the SLYM

Reaction	Background	Problematic	Severe
All (a)	50 1,000	10,000	100,000
DS (b)	1 100	5,000	10,000
BL (c)	20 100	500	1,000

Note: (a) refers to the risk analysis for all recognized reactions except DS and BL; (b) dense slime growths are indicative of a tight biomass being formed commonly at the oxidative-reductive interface and causing restrictions water flows and quality; and (c) relates to terminal black liquid, BL, reaction that do occur when conditions are reductive with a relatively high organic burden.

11.5 Product name: HAB- BART Pcode: bacterial biotester

These bacteria are essentially the “organic busters” and play major roles in the degradation of organics (such as in bioremediation of hazardous waste sites). This bacterial biotester specifically determines the activities of the heterotrophic bacteria using methylene blue as the indicator of oxidative (blue) and reductive (clear) conditions. Here the biotester always starts in an oxidative state as blue and then moves to a clear state when the bacteria become active. There are two major reactions which separate the bacteria into dominantly oxidative (aerobic) as the UP reaction; and reductive (anaerobic) conditions as the DO reaction. Up reactions begin in the base of the biotester and the blue shifts to clear from the base upwards. Risk analysis for this reaction would primarily relate to aerobically active

bacteria. DO reactions commonly begin just below the floating ball when transient clearing may first occur and then shift back to the original blue color. As the anaerobic activity continues then the reduction of the methylene blue becomes more permanent and a clear zone now forms below the ball and moves downs. There are two risk analyses in Table 11.5.1 recognizing the different nature of the two reactions.

Table 11.5.1 Risk Analysis for the Investigations of the HAB

Reaction	Background	Problematic	Severe
UP (a)	10 1,000	50,000	100,000
DO (b)	1 100	5,000	10,000

Note: (a) UP reactions are a clear signal that the HAB- are dominated by aerobic (oxidative) activities and these bacteria tend to grow prolifically within the biofilms and biocolloids meaning that high populations can be found in degradable organic-rich waters; and (b) dominated by reductive (anaerobic) activities that would mean that the populations may be smaller but capable of causing problems from the daughter products such as fatty acids (causing the pH to shift to more acid) and gases (particularly carbon dioxide, hydrogen, methane and nitrogen) which then can become perched with a foam rich biomass and cause changes in the hydraulic flows through the impacted region.

11. 6 Product name: APB- BART
Pcode: acidogenic biotester

Under organic-rich reductive conditions it is likely that fermentative bacteria will dominate the growing biomass

generating daughter products that include fatty acids and gases. Under these circumstances the normal outcome is at least a temporary drop in pH into the acid range if the biomass buffers the acidity back to neutral (DY⇔). Where buffering occurs then there would be a broader spectrum of heterotrophic bacteria present and the biomass could become more challenging to the engineered system. If the fermentative function generates stable acidic pH then this is likely to have a traumatizing effect of the biomass which would then become less active. In the acidogenic biotester the objective is simply to determine fermentative activities under reductive conditions. Here the pH indicator turns yellow but the reaction is clouded by the generally intense bacterial activity. Hence the yellow color is commonly made dirty with these bacterial activities. The only time that a bright yellow reaction will be observed is when the fermentative activity focuses around, or just below, the ball. Here the DY reaction would be declared once the bright yellow reaction appears stable around or below the ball. In such cases there is a descending DY reaction until the whole tester has a uniform yellow color. Buffering is a condition where the DY reaction now reverts to the purple colors as the acids are bacteriologically neutralised. This buffering commonly begins in the base of the tester or around the floating ball where there is an abundance of headspace (diffusing) oxygen. This tester is very relevant to sites where there is some evidence or potential for corrosion since APB- will cause an acidulolytic form of corrosion which can be as significant as the hydrogen sulfide induced electrolytic corrosion influenced by the presence of sulfide producers (e.g. SRB-).

Table 11.6.1 Risk Analysis for the Investigations of the APB tester

Reaction	Background	Problematic	Severe
DY (a)	10 1,000	50,000	100,000
DY ⇔ (b)	1 100	5,000	10,000

Notes: (a) is the generation of a "dirty yellow" reaction which may begin around the ball or further down in the culturing sample and indicates the bacteria are anaerobic functioning within a reductive environment as an aggressive biomass; (b) the symbol "DY⇔" indicates that there is a buffering of the pH once fermentation has dropped back into the more neutral values and so the biomass essentially acts to correct the pH which is seen returning towards neutral. In the latter (buffering) event a relatively small but adaptable bacterial consorm can be involved.

11.7 Product name: N- BART
Pcode: nitrate biotester

Nitrifying bacteria are unique within the bacterial kingdom because of their ability to oxidize ammonium (from the reductive anaerobic degradation of proteins) into nitrate. Nitrate, when present in potable waters, can present a threat to the very young and the old and so strict regulated guidelines are enforced to limit nitrates (and hence the activities of nitrifying bacteria). Nitrifying bacteria are relatively slow growing and often require long adaptation times before they become active. In the oxidation of ammonium through to nitrate these bacteria create very demands for oxygen. This has been a problem in the

biochemical oxygen demand (BOD) test since its inception. Because this test takes five days of incubation there is the potential for any ammonia to become oxidized to nitrate with a very significant demand for oxygen. Such demands distort the functioning and precision of the BOD test and inhibitors have been applied to control this nitrifier-influenced excessive oxygen demand. The nitrate biotester is, in its simplest form, a presence-absence test for nitrifiers. It is possible to semi-quantify the nitrifier activity by undertaking the test on 10^{-1}, 10^{-2}, 10^{-3} dilutions. After five days incubation the test for the presence of nitrite is used to measure the activity of the nitrifiers (Table 3.2.7.1.). Nitrite is used as the prime detection of nitrification since it was found that the nitrate generated by the nitrifiers (group 2) was commonly rapidly reduced to nitrite (as a result of denitrification). Essentially the nitrate biotester focuses on the emergence of nitrite during the test as a result of the activities of the nitrosofiers (group 1). In the application of a dilution series it is possible to now semi-quantitatively predict the populations (e.g. in sanitary wastewater treatment plants).

11.8 Product name: DN- BART
Pcode: nitrite biotester

This tester is called the nitrite tester since the initiating chemical for the detection of denitrification is nitrite and the product of activity is nitrogen gas that collects temporarily as a foam ring around the ball. Detection of denitrification is totally dependent upon the recognition of this foam ring when it occurs during incubation. Populations are predicted based upon the time lapse generated and interpreted (see Table 5.6.4.1). Essentially there are circumstances when these denitrifiers do remove nitrate (and therefore make the water "safer" but nitrification is a reductive event that

occurs within the biomass. Secondary consequences of this activity can be the generation of foam initiated plugging that, at least temporarily, interferes with hydraulic flows. Additionally there are the daughter products of these reductive biomass activities that can be problematic for the functioning of the ecological system. Since the aerobic biomass will input a primary demand on intrinsic oxygen in the eco-system there will be a substitution of nitrate or nitrite for oxygen under reductively-stressed conditions. This would mean that denitrifiers will become more active, or even dominant, within a biomass transitioning from oxidative to reductive conditions. One major event where the denitrifiers do dominate is in the movement of sanitary wastewaters. The sequence that causes this to happen would be: (1) anaerobic degradation of the wastewaters with proteolytic releases of ammonium; (2) movement of wastewater into an oxidative environment where the aerobic biomass that assimilates the total and fecal coliform bacteria; (3) nitrification of the ammonium moving into the aerobic biomass generating nitrites and nitrates; and (4) denitrification of these nitrates and nitrites once the wastewater now re-enters reductive zones. Here the denitrifying bacteria will become a greater part of the population and be indicative of the movement of products from the breakdown of the wastewater. Table 11.8.1 illustrates the risk of this occurring using Table 5.6.4.1. as the generator of the populations.

Table 11.8.1. Risk Analysis for the Investigation of the DN- (denitrifiers).

Reaction	Background	Problematic	Severe
FO (a)	0 100	1,000	10,000

Note: (a) is a transitory phenomenon in which the foam ring (FO) is commonly only observed for one to two days before dissipating.

11.9. Product name: FLOR- BART
Pcode: glow biotester

There are two significant reactions that are recognized as relating to specific (health) risks. These are both ultra violet light fluorescing pigments that for a period of time around and below the floating ball in the incubating tester. Both relate to species of *Pseudomonas* with the pale blue (PB) relating to *Ps aeruginosa* group and the other being a greenish yellow (GY) generated by the *Ps fluorescens* group. Here the PB reaction which usually occurs in the top 20mm of the culturing fluids for just two to three days in indicative of a potentially serious health risk. For the GY reaction there is a lower health risk and this group tends to be often associated with intense biodegradative activities associable with the oxidative breakdown of specific organics such as the total petroleum hydrocarbons or natural gases.

Table 11.9.1 Risk Analysis for the Investigations of the FLOR

Reaction	Background		Problematic	Severe
PB (a)	0	100	500	1,000
GY (b)	0	500	1,000	5,000

Note: (a) involves the U.V. glowing with a pale blue color for the top 20mm around and below the floating ball with the glow persisting only two to three days and confirmation of the presence of *Ps aeruginosa* strains can be achieved using 1ml of the culturing fluid taken immediately beneath the ball using a sterile Pasteur pipette; and (b) involves a

more persistent (commonly 5 to 10 days with inception on day 2 or 3) green-yellow (long wave length) U.V. glow that can extend half way down the biotester.

11.10. Product name: ALGE- BART
Pcode: microalgae biotester

Micro-algae are a major biomass in surface waters and are commonly dominant the phytoplankton growing in surface blooms. The risk in this case relates to the algal biomass that can interfere with the natural and engineered treatment processes. These blooms do also extend into soils where summers blooms can occur (although maybe not observed or recognized as such. Water wells can be contaminated not only by recharges from nearby surface waters but also from algae moving down with localized recharges of water moving through fractures and porous formation structure outside the effectively grouted zones. Some of the micro-algae (cyanobacteria) can present the DG reaction and this maybe indicative of a potential health risk primarily from the generation of toxins.

Table 11.10.1 Risk Analysis for the Investigations of the ALGE

Reaction	Background	Problematic	Severe
All (a)	0 100	1,000	50,000

Note: (a) refers to all six reaction types and the populations predictions are generated using Table 3.8.2.1. This risk relates only to surface water quality and shallow groundwaters being recharged from surface waters where there may have algae infesting the wells with the recharge waters.

Chapter 12

Innovative BART tester Applications

12.1 Using BART testers in Unusual Soils, Waters, Wastewaters & Other Water–Containing Materials.

This is possibly the most challenging chapter in the Standard Methods for BART testing since this will recount the many investigations where the results contradict the standard text book accounts. It has to be remembered that a classical text book along with dogmatic thinking tends to follow narrow (linear) pathways with the reader being encouraged but never to the side or forwards. Progress in science and technology is made by looking sideways, forwards, upwards and downwards and listening to the language of Nature. Science is not as described in text books but surrounds us everywhere that we look, hear, touch, and feel.

12.2 Bees, foulbrood infection

Define: Bees are prone to infection by spore forming *Bacillus* species. These bacteria infest the bees within a colony causing high rates of fatality (American foulbrood)

Apply: Aseptically homogenise 10g of suspected bees in a sterile blender with 90ml sterile distilled water. Heat suspension to 80°C for ten minutes; cool quickly, Inoculate three HAB- testers with 15ml of homogenate. Incubate at room temperature.

Consequence: If spore formers are significantly present then an UP reaction would be observed within three days. Use the first tester to go positive to provide confirmatory culture.

12.3 Bioremediation

Define: Environmental concerns have raised in the last two generations over particularly the impact of recalcitrant organics such petroleum hydrocarbons on the potable quality of groundwaters. This has resulted in the growth of engineered bioremediation based on the premise that oxidative processes will causes the biochemical degradation of the organics-of-concern. These degradative functions are, for the most part, bacteriological.
Apply: Of the testers it is the HAB- BART that is the most suitable since it does detect oxidative (aerobic) functions as UP reactions and, in effective bioremediation processes, the bacteriological population can become very large and active (generating short time lapses and UP reactions). If the TPH (total petroleum hydrocarbon) do not exceed 300ppm then the standard techniques for soil or water can be followed (see table 5.1.1.) but if there is a higher TPH then the methylene blue needs to be dissolved in the cap using 1ml of sterile distilled water and this added to 14ml total volume in the tester. Room temperature can be used for incubation and the VBR system is recommended to achieve precision.
Consequence: Compared to control where there is no significant TPH present the testers should generate UP reactions in time lapses much shorter than controls indicating at least increases in the predicted population.

12.4 Black goo in oil

Define: Black goo particularly in oil in pipelines originates in part from bacteria literally "mining" the water out of the oil and containing it within black anthracenes for protection for the growing biomass.
Apply: Aseptically take 50g of black goo, add 50ml of sterile distilled water, and blend in a sterile homogenizer until suspension evenly dispersed. Add the 100ml suspension to sterile 250ml graduated cylinder and leave static at room temperature overnight. Water will collect under the oil (commonly 10 to 30ml). Using a sterile 25ml pipette withdraw the available pooled water avoiding any black particulates. Dilute 10ml of water with 90ml of sterile distilled water, and us 15ml aliquots for duplicates of HAB-, APB-, SRB-, SLYM- testers. Incubate at room temperature. Confirmation of bacterial activity would be both of the duplicates giving: HAB- probable DO reactions in less than two days; APB- giving a DY reaction in less than three days that may buffer out; SRB- BT reaction in less than four days (BB not important); and SLYM- giving CL in less than two days.
Consequence: Particularly the HAB- reaction UP would signify intense bacterial activity and the aerobic nature could relate to the use of cathodic protection for the pipe leading to enhanced electrolysis supporting the generation of oxygen at some locations.

12.5 Black layer in soil

Define: Coring into soils and golf greens sometimes shows a defined lateral black layer commonly between 5 and 45mm down from grade.
Apply: Remove aseptically soil from the black layer and use 1.0g in 14ml sterile distilled water for each of

the duplicate testers: HAB-, IRB-, APB-, and SRB-. Incubate at room temperature.

Consequence: black layer will commonly give DO in HAB- in less than two days with secondary blackening with reduced organic compounds; IRB- will give a final BL reaction in less than eight days; APB- will give a DY reaction within two days and SRB- may give a BT reaction within five days.

12.6 Black smokers

Define: black smokers are found in deep ocean hydrothermal vents and seeps that are exuding black hot water into the ocean. Water temperatures inside a smoker can reach 200°C or more. This water would also be high in salt.

Apply: Take water sample suitably to allow BART testing when sample is brought to laboratory (on-ship or on-shore). Prepare duplicates of HAB- and SRB- testers. Run one set at room temperature and the other at blood heat.

Consequence: HAB- should give DO reactions in less than one day and the SRB- should give a BT reaction closely followed by a BB. Both tester types are likely to turn black due to the presence of iron sulfides (SRB-) and reduced organic compounds (HAB-). It may be expected that the blood heat tests will generate positive reactions quicker than room temperature tests.

12.7 Cancers

Define: Cancers are generally considered to be caused by tissue cells that become unstable and, commonly, reproduce in dysfunctional manners. It is possible that several cancers are, in reality, the product of bacterial infestations within those tissues. For example, the

recent linkages between stomach cancers (ulcers) and *Helicobacter* species. In the growth of bacteriology there has developed since 1884 (false) assumptions that agar media detect all significant bacteria. It is proposed that the tester systems functioning at blood heat could provide an alternative method for detecting significant bacterial infection associable with cancers.

Apply: This is a theoretical example using the SRB-tester (laboratory version) to examine for the activity of SRB that are possibly suspected to be associated with colon cancer. Here voided fecal material should be homogenised aseptically using 10g fecal material in 90ml sterile distilled water. 15ml of the homogenate is now used for each SRB-BART tester incubated at blood heat. Observe daily for BB or BT reactions.

Consequence: BT reaction within three days or BB reactions in four days would indicate very active SRB in the feces which could possibly be linked to colon cancer. If these reactions occur after an additional three days or more of incubation then this may be normal SRB activity levels.

12.8 Clay, from kiln

Define: Clays are dried in an oven at high temperatures to remove most of the water and create a dry product. These kilns operate commonly at greater than 400°C and this does not remove all of the water but theoretically kills all of the microbes. This may not be true for some of the spore forming bacteria that appear to be “protected” when in the dried endo-spore form.

Apply: Aseptically remove cooled clay that has just gone through the kiln and place in a sterile container. Make sure that the clay has cooled down. Take 1g samples and add the following testers (use the rolled out ball into the cap technique): HAB-, SRB-, and

SLYM-. Make up to 15ml in the tester with sterile distilled water. Incubate at room temperature and observe for reactions bearing in mind that the kiln-heat should have killed all of the microorganisms that were resident in the clay. In this test there may be a period during which the clay remains in suspension but this should end when the clays settles out to the base of the tester. Some electrically active clays may show particulate movements involving floating and relocation.

Consequence: If the clay kiln had been effective then there should be no reactions. However, if endo-spore formers had survived than there would be a reaction in at least one of the tester types within ten days of incubation. Note that the surviving endo-spores did surviving the heating process then there would be delays in the time lapse as the spores rehydrate and germinate.

12.9 Clay, separator

Define: Clays are separated from impurities by using a separator that allows the clays to become concentrated. This may involve physical and chemical process but inadvertently may trigger intense microbiological activity that may cause very significant biofouling that could then cause process failure. It is not generally recognized that during solution process for separating out clays bacteria can cause major quality and flow problems.

Apply: In the clay separation process there would be both liquid (usually highly turbid) and suspended solids. BART testing would be focussed on the turbid liquids using the premise that this would support the biofouling bacterial agents. The protocol would involve taking 1.5ml of the liquid sample and dispense into the tester followed by 12.5ml of sterile distilled

water. Preferred testers would be the HAB- and APB- testers with the reagent in the caps (methylene blue for HAB-; and bromocresol purple for APB-) pre-dissolved in 1ml of sterile water and then added to the charged tester. Incubation would be at room temperature with daily readings until positive or day ten.

Consequence: For the HAB- tester a reaction in less than three days would be considered significant for a biofouling risk in the separator with the UP reaction indicating oxidative conditions and DO for reductive conditions that could lead to the generation of blackening in the bottom half of the tester due to reduced organic compounds. For the APB- tester the generation of the DY reaction would mean that the pH of the process may be becoming too acid with secondary corrosion risks.

12.10 Clouds

Define: Clouds form major factors in the weather controlling whether it will be hot or cold, wet or dry. It is generally recognized that clouds do contain water droplets and generally it was thought that the water gathered around dust nuclei. More recently clouds have been thought that the water forms around bio-nucleating particles which were essentially microbial and/or their products.

Apply: Sampling within clouds is a challenge since there needs to be a means of condensing the droplets from clouds (where they are bio-nucleated) into liquid samples. This needs to be done with a minimum disturbance to the cloud which, although large, are very fragile while at the same time being dynamic. Here the most suitable method may be to float within the cloud in a dirigible (airship) and remove some the air using an aseptic vacuum system. Here the air inside

condensers causing the droplets to condense into liquid water. Such water should be tested using 15ml samples in HAB- BART testers incubated at room temperature. **Consequence:** If the clouds are active with bacteriologically induced nucleation within the droplets then an UP reaction can be expected to occur within two days. Conditions within the cloud should be highly oxidative and support respiratory forms of metabolism. In the rare event that DO reactions are recorded then this would mean the sampled cloud was under stress (reductive fermentative) state and may also be less stable.

12.11 Concrete, curing

Define: Concrete is viewed as a physico-chemical product that gradually hardens (cures) over time. The curing time commonly of 28days would indicate that there could be a biological function for the "curing" as a bioconcretion function. Bioconcretions are very common in Nature where microorganisms render predominantly inorganic structures into habitable protection for the incumbent biomass. Rusticles are good example of bioconcretions that are complex porous layered structures in which several bacterial communities (consorms) are active but at distinctly separate locations. Concrete is different as a heterogeneous mixture of inorganics that mature into a stable structure. Comparing Portland cement prepared under sterile conditions with a non-sterilized cement using tap water it was found that the compression strength of the sterile cement was 27MPa while for the non-sterile it was 33MPa in triplicated trials. This further suggests a potentially significant role for bacteria in the curing process.

Apply: Using 1g samples and SLYM-, HAB- and SRB- testers using sterile distilled water to make the volume up to 15ml with incubation at room temperature, these testers were observed daily or place in VBR systems. Note that the HAB- tester system should have the methylene blue pre-dissolved in the cap using 1ml of sterile distilled water and then added to the 1g sample plus 13ml of sterile water to make a final volume of 15ml.

Consequence: SLYM- testers usually generate a dense gel (DG) as the first reaction followed by clouding (CL) and possibly foam (FO); SRB- testers will usually generate copious gas that should form into a foam (FO) with the BB and BT reactions depending upon the sulfate and organic contents of the sample; HAB- will normally generate UP reaction and there may be fluorescence around the ball. These reaction indicate that bacterial are active and time lapses will shorten as activity increases from samples taken later during the curing process.

12.12 Drywall, sheetrock

Define: Drywall (or sheetrock) is a common construction material used for smooth finishes to internal rooms under dry conditions. Drywall is actually gypsum layered between cellulosic (paper, cardboard) layers. While the drywall stays dry then the sheets are stable. Once there are persistent damp patches then microbiological deterioration can occur. If the damp patches persist then molds (fungi) are likely to thrive in the porous structures leading to spores forming on the outside of the infested drywall. If the moisture saturates the drywall to a greater extent (e.g. during a flooding of the home) then various bacteria can actually compromise the gypsum. Two

bacterial consorms (SRB- and DN-) have been found to actually "digest" the gypsum causing the drywall to collapse.
Apply: Investigations should be applied to those patches of drywall that are in state of physical collapse due to losses in structural integrity of the intrinsic gypsum within the panel of drywall. To do this, aseptically remove 1g samples of the gypsum from the drywall. Using the SRB- and DN- laboratory testers, roll the BART ball out by the standard method into the cap; add the gypsum sample; add 14ml sterile distilled water, return ball and cap. Incubate at room temperature.
Consequence: SRB- tester is likely to go a BT reaction if the drywall had become soaked in a flood including wastewater; BB reaction is likely if the drywall had become very damp for a long time but it did not involve flooding. After four to six weeks if there is any SRB- bacterial activity then the gypsum sample will be seen to disappear but there may be black iron sulfide granules lying where the gypsum had been. DN- may or may generate foaming (FO) but will generate clouding from bacterial activities. In six to twelve weeks the gypsum sample may also disappear. Note that there is a possibility of mold (fungal) growth which will appear as a black furry ring around the BART ball and there might be some growth over the top of the ball. Here the significant fact is that these two bacterial consorms have the ability to degrade the gypsum.

12.13 Encrustation

Define: Encrustation is the descriptor for a biomass usually growing on a surface and has a high inorganic content dominated by iron or carbonates. It generally is heterogeneous in form, porous and structurally

resilient. These types of growths are seen naturally in fractures, on surfaces over which water is passing, and in equipment such as heat exchangers. These types of growth can become associated indirectly with corrosion and losses in plant efficiency.

Apply: Sampling is relatively easy since encrustations retain form even when the water is drained out of or simply drained from the site of infestation. It should be noted that old encrustations that have been drying out of the water environment will slowly lose their bacteriological activity. Suitable test methods most easily employ 1g of the encrustation that is applied to the tester after the ball has been removed. Recommended testers include the IRB- (for iron related bacteria), SRB- (for sulfide producing bacteria), and APB- (for the acid producers). Incubation is commonly at room temperature and the diluant can be either sterile distilled water (if there is a low chemistry in the water) or, in the event of total dissolved solids content, >5,000ppm then sterilised water can be used from that environment.

Consequence: IRB- tester is likely to generate a WB (white base of carbonates) within twelve hours of the test starting if the encrustation has high inorganic carbon contents. If there is significant iron then the oxidative ferric reactions leading to one or more of BG, BC and BR reactions. If there is large mixed bacterial consorm including enteric bacteria then RC, GC are likely to occur before terminating in a BL reaction. SRB- giving a BT reaction would mean that the encrustated biomass has high organic contents while the BB reaction would indicate that there were sulfate reducing bacteria and active. If the APB- tester gives a DY reaction then that would mean that the biomass was reductive at least in part with the generation of fatty acids. Examining the surfaces under the encrustation when an iron-alloy is involved will

indicate the type of corrosion. For example, shallow dishing would indicate acidulotyic corrosion by APB-. Shallow local pitting with blackening would indicate electrolytic corrosion by SRB with BT tending to support widespread shallow pitting and BB deep pitting likely yo lead to perforation events.

12.14 Filter, air

Define: Filtering air is achieved using a fine porous structure that entraps the solid particles (e.g. dust, mold spores) and do not allow the air to pass through. Inevitably the pores become blocked up by the particles causing increasing back pressures to force the air through.

Apply: Air filtered from relatively dry air is less likely to contain bacteria but more likely to contain molds. Moist air however remains likely to contain a variety of bacteria. The most convenient way to check this is remove a part of filter (e.g. 10 sq cms) and immerse in 100ml of sterile distilled water for five minutes. Agitation would cause the particles to detach from the filter and go into suspension. 15ml of the suspension should be used in each tester (SLYM-, HAB-, and SRB-) with the potential to duplicates. Incubate at room temperature and observe at least daily.

Consequence: CL reactions from the SLYM- tester are most likely to indicate the bacteria content in moister air while a fuzzy black ring (not recognized as a standard reaction) would indicate the growth of mold spores. HAB- tester data would indicate whether these bacteria active in the dust are aerobic (growing in moist air as an UP reaction) or anaerobic (emanating from moist reductive regions as DO reactions). SRB-tester would only give positives if there are particles from reductive conditions that are deep seated (BB

reaction) or associated with moister more organic conditions (BT reaction).

12.15 Filter, membrane

Define: Filter membranes have the function of removing smaller particles such as bacterial cells. These utilize pore diameters do not to allow these bacteria to pass. Diameters commonly used are 0.45 and 0.22micron diameter pores. Theoretically no particles larger than those diameters should pass through however that assumes these particles are robust and rigid, Reality is that any flexibility may allow some of these particles (cells) to pass through the membrane. Additionally smaller particles (such as biocolloids) that are commonly flexible will also pass through. These particles often collect on the downstream side of the membrane and provide nutritive environments for the survival and growth of microorganisms downstream of the membrane. Filter membranes are considered excellent methods for trapping bacteria on the upstream surfaces of the membrane for subsequent cultural and biochemical analysis. Downstream flows from the membrane are generally considered sterile although these can be high in biocolloidal materials. The challenge is primarily to separate the upstream surface (trapping microorganism) from the lower downstream surfaces (gradually fouling with biocolloids and microbial activity generated from downstream sites.
Apply: Functionally effective membrane filters should remove all viable cells of concern from the water passing though the membrane. The prime test must therefore examine this downstream water for bacterial content using the premise that the water would have been filtered and therefore free of microorganisms. This test is a replicated (x5) using both the SLYM-

and the HAB- testers. Total volume required is therefore 150ml using incubation at room temperature and daily observations until positivity is recorded.

Consequence: Ideally all ten replicates after ten days should remain negative meaning that the maximum population of detectable bacteria should not have exceeded seven cells per litre (7pac/L). Higher populations would be detected by one or more testers then the types of reaction observed become critical. Of serious concern would be DO reactions using the HAB- tester and DS reactions on the SLYM- tester since these would indicate that the biocolloids that are sheering from the downstream side of the filter. Here, anaerobes (DO) and dense slime formers (DS) could be present. PB and GY reactions on the SLYM- tester are also serious signals since these shows the presence of active *Pseudomonas* species.

12.16 Gypsum

Define: Gypsum is a common natural geological product composed of calcium sulfate which has a number of important uses (see drywall above). Under unique high salt alkaline conditions associable with the presence of the oxidative-reductive interface in groundwater, gypsum can form as deposits that can effectively plug impacted water wells. This protocol defines the occurrences of microorganisms in the synthesis and dissolution of gypsum.

Apply: This protocol examines both the bacteria likely to be present in the gypsum and the potential for those bacteria to degrade the gypsum under reductive conditions. For this each tester would require 1g of gypsum sampled aseptically following the protocol adopted for soils. 14ml of sterile distilled water can be used as the diluent or steam sterilized groundwater from the site where the gypsum was recovered. Testers

should include SRB-, DN-, SLYM- and HAB. Incubation would be at room temperature for ten days followed by weekly examination for the dissolution of the solid gypsum sample.

Consequence: For the bacterial content of the gypsum the SLYM- and HAB- tester data should be used. SLYM testers may show DS but always CL possibly with some FO by day five while the HAB- may show diffuse types of UP or DO reactions. Bio-dissolution of the gypsum may be seen in clouding (CL not recognized as a reaction) in both tester types with a possible BT or BB reaction in the SRB- tester. While this may occur in less than seven days the testers should be observed weekly for the bio-dissolution of the gypsum sample that can occur in three to five weeks for the SRB- tester and take twice as long in the DN-.

12.17 Ice, cores

Define: Ice is considered to be frozen water that is too cold for any life to occur and, certainly, not be capable of growth. Reality is that there are bacteria that can either synthesize ice even at temperatures above conventional freezing, or produce polymeric antifreeze compounds that allow the bacteria to remain active in a liquid environment at temperatures well below freezing. Ice cores are taken from "old" ice that may have been relatively stable for even millennia. Because of this stability ice cores are used to examine old ice fields to determine changes that had occurred when the ice was laid down. Reality would be that the ice would minimally have been infested with microorganisms at the time of freezing and this would have allowed a slow but ongoing microbiological activity.

Apply: Bacteria are capable of surviving as ultramicrobacteria, suspended animates, or spores for

prolonged periods in ice let alone remaining active vegetative cells. Ice cores are not designed to be extracted aseptically and so the outside of the core will be potentially contaminated. Any microbiological investigation must therefore work the central (25%) core which would have been less affected by the coring process. For the sample now refined under aseptic conditions the temperature for thawing should not exceed 8°C. This would be to minimise the temperature rises associated with the thawing and manipulation of the sample. Once the sample is liquefied then there is a need to leave the sample for seven days to allow the bacteria time to adjust to the liquid state. All incubation of the charged testers should be at refrigeration temperatures (4±2°C) with care taken that the testers and such fluids as may be applied being pre-cooled to those temperatures to minimise cultural shock. At least 90ml of sample needs to be collected to allow triplicate analysis using both the HAB- and SLYM- BART testers. Incubate at 4±2°C in a refrigerator ensuring that all of the testers have good air circulation. Examine daily for reactions for twenty eight days or until the tester generates a positive reaction.

Consequence: Because of the low incubation temperatures involved there could be extended time lapses as the bacteria adapt to conditions in the tester. For the HAB- either UP or DO reactions are possible followed under very reductive circumstances by a partial BL (lower third to a half); while the SLYM- tester may generate CL reactions possibly preceded by TH or DS and followed by BL (lower third to a half of the tester). No correlations have yet been developed for the relationship of time lapse to the intrinsic population in the ice.

12.18 Ice, gas hydrates

Define: Gas hydrates are deep-ocean structures embedded into the sea floor. They are dominated by an ice – methane (1: 8±2) matrix that forms the gas hydrates (clathrates) as one of the largest sources of natural gas known on the planet. These clathrates appear to remain stable up to 7±2°C with the ice matrices remaining in-place. Evidence suggests that the bacterial consorms involved in gas hydrates are capable of synthesizing ice at these higher temperatures. These appear to be heterotrophically active bacteria (HAB-) or concretion forming (IRB-). Coring into the top two meters of the clathrate allows examination of the bacterial capping on the clathrate.
Apply: Ice cores could be melted with care slowly at 12±2°C and then the incubation would be at room temperature 22±2°C using 15ml sample volumes in three HAB- and three SLYM- testers. Observations can be using the VBR system set to record every 15minutes or visually daily.
Consequence: Generally the HAB- testers generate UP reaction near the surface of the capping (0 to 100mm) and DO reactions in the deeper regions of the cored gas hydrate. However at depths as great as 2m there can still be oxidative zones where UP reactions can also occur. SLYM- reactions tend to be dominated by CL with follow up BL commonly in the lower third of the sample and possible limited FO.

12.19 Ice, glacier

Define: Glacier ice is generally ice that has formed by a process of packing down (under increasing pressures with depth) incorporating such particulate material that may have been deposited into the ice as it formed. This ice is subjected to movement along with fracturing and differential melting

along particulate-rich fronts. This would mean that the crystalline matrices within the ice would be more vulnerable to physical stresses than some other forms of ice. Additionally the temperature of glacier would tend to be more variable particularly if above -18°C then there would be a greater potential for bacterial activity. The common presence of ice worms in some glaciers could be indicative of bacteriological activity that is then providing the feedstock for the worms.

Apply: Glacier ice tends to be variable in terms of physical form and color (e.g. grey, blue, black, ferric red). Greater bacteriological activity is likely to be associated particularly with these colored bands. Samples taken from glacier ice should be kept in a freezer (lower than minus 18°C) until ready for investigating. Here the ice can now be thawed at room temperature until the ice has completely melted and then immediately tested. Incubation would be at room temperature (22±2°C) and in a refrigerator (4±2°C) until the tester generates complete reaction sets or for ten days. 15ml of melted ice would be used for each tester with HAB-, SLYM-, IRB- and SRB- being recommended.

Consequence: HAB- testers should normally give UP reactions unless the ice sample has come from deep inside the glacier in which case DO will occur if conditions are more reductive; SLYM-testers would commonly show CL but this could be preceded by DS or TH; IRB- testers are likely to generate CL followed by BG or BR reactions unless the ice sample is from a reductive region in which case FO reactions may occur first; and SRB- reactions are most likely to be BT if there is organics are present with BB only occurring in the event of a sulfate rich environment that was more reductive in nature.

12.20 Ice, pack

Define: Pack ice essentially floats on water and is often seasonal in its occurrence depending upon the local freeze-

thaw cycles and the movement patterns for the ice pack. This form of floating ice easily bioaccumulates material perching particularly on the underside of the ice. This becomes very significant when the tidally influenced pack ice become exposed to effluents originating from sanitary landfill or wastewater treatment operations. Maximum bacterial activity is generally associated with the black viscid voids that permeate the pack ice at points where there has been intrusions of these waste effluents.

Apply: In the evaluation of the bacteriology of pack ice that is showing signs of having been compromised the ice samples should be kept in foil or freeze resistant plastic wraps in a freezer (lower than minus 18°C) until ready for investigating. To investigate particularly the blackened track ways within the ice then these can be eased out of the ice sample using sterile scalpels and spatulas. Incubation would be at room temperature (22±2°C) and in a refrigerator (4±2°C) until the tester generates complete reaction sets or for ten days. 15ml of melted ice would be used for each tester with HAB-, SLYM-, IRB- and SRB- being recommended.

Consequence: In the event that bacteria activities are detected in one or more of the testers then the time lapse (predicting the population) and the reactions can be significant. HAB- testers are likely to generate UP reactions normally but DO reactions if conditions in the pack ice are reductive. In the latter case it is also likely that regardless of reaction type a BL will also be generated rising one third to half way up the culturing sample. SLYM- testers generally will initiate with a CL that might be preceded by DS but almost certainly will be followed by a BL. IRB- testers may begin with FO followed quickly by CL and then reactions reflecting the local environmental conditions (reductive, RC, GC, and BL; oxidative, BC, BG and BR. SRB- testers are most likely to give the BT reaction followed by BA in conditions where there has been septic waste entrained into the pack ice.

12.21 Mold spores

Define: Mold spores are durable reproductive elements commonly generated by fungi. These spores are often very noticeable as furry growths on surfaces while the fungal biomass remains growing underneath that surface in relatively porous media. Mold spores are moderately resistant to heat shock compared to vegetative microbial cells and 72°C for 5 minutes of exposure time will eliminate much of the interferences from such vegetative activities. Molds are a problem under conditions such as dampness with perched water patches (e.g. water-compromised drywall). Some fungal spores can trigger lung infections.

Apply: Two common sources of mold infestations are damp patches which display black spots of growth within confined areas or the air which does support mold spores which are suspended in the circulating air currents. For the former then a sterile swab can be used to remove some of the growths which can then be dispersed in 15ml sterile distilled water. Spores suspended in air can be recovered by filtering the air (e.g. 1 litre) through sterile 0.45micron membrane filter. Here the membrane filter should be removed from its holder and immersed in 15ml distilled water for fifteen minutes. In either case approximately 15ml of suspension is obtained once the swab or filter is removed. Using a sterile 25ml glass tube as the holder for the suspension then the contents are heated to 72°C for 7 minutes allowing 2 minutes for warming up the suspension. Cool down quickly by immersing the tube in cold water. Use 5ml of the heat-treated cooled suspension in each of two SLYM- testers with sterile distilled water and incubate at room temperature for fourteen days.

Consequence: If molds do grow in the testers then the first signal of growth would normally be a woolly type of growth (commonly white) under and around the submerged ball. This would be followed after 2 to 4 days by the evidence of

mycelial growth over the top of the ball which, concurrently, would exhibit black particles formed by spores growing on the exposed mycelium.

12.22 Mud

Define: Mud may be considered as wet, soft earthy matter that forms on the surfaces of soils and shallow ponded waters. Essentially muds are mixed amalgam of soil particles, organics and active biomass. As such the muds can be microbiologically very active which can be influenced by the oxidative-reductive potential (ORP), the exchange capacity of components and the quality of the water.
Apply: Using Table 5.1.1 as the guide (select loam as the first option with clay as an alternative if it is known that the mud has high clay contents rendering it slippy). Testers for mud should include HAB-, SRB-, APB- and SLYM-. Incubation should be at room temperature and observations made daily or every 30minutes using the VBR system.
Consequence: HAB- will trigger as UP reactions if the mud has an open structure and DO reaction if relatively tight. SRB- will show clouding (not a recognized reaction) that could include particle cycling if there is any gas formation in the sample. High organic muds are likely to give the BT reaction and dense muds with higher sulfate content are likely to give BB reactions. If the mud is reductive with the groundwater having a high organic content then the APB could give DY reactions due to fermentation with fatty acid generation but the acid reaction is likely to be buffered as the fatty acids are utilized by SRB- or CH4- (methane producing bacteria).

12.23 Oil, crude

Define: Crude oil consists of longer chained hydrocarbons (C_xH_y) which have been generated biochemically with

bacteriological influences under extremely reductive conditions. Crude oil along with natural gases and coals reflect the stripping of the organic matter generated primarily in the surface biosphere down to elemental carbon (coal), natural gas (CH_4) and petroleum hydrocarbons (C_xH_y). Crude oil become a reserve that has limited stability depending upon the fractions of volatile hydrocarbons that will diffuse upwards, and entrained water that then become the site for ongoing bacteriological activity. Essentially the entrained water becomes a "desirable" component in the oil that is mined and bound within extracellular polymeric substances by the bacteria within matrices of asphaltenes. The greater the water contents in the crude then the greater the potential for bacteriological activity in that oil. In old wells then the water may become so abundant that it separates out as "produced water" and has a high bacteriological content.

Apply: Crude oils are predominantly oil with a small fraction of water that will sometimes increase over time. To investigate the oil for bacteriological content the first step is to take a small volume of oil (100ml) and emulsify in a larger volume of sterile distilled water (e.g. 1,000ml). This oil-water mix needs to be emulsified using a sterile blender until the oil is dispersed in the water. Leaving the oil-water mix at room temperature will cause the oil to gradually rise to the surface if it has a lower density that water. Much of the bacterial biomass in the oil will be dispersed by the emulsification process into the water where it may tend to rise to just under the oil. After 24 hours extract the upper 100ml of water perched below the oil (and not impacted by any observable oil droplets). This is the most probable site for the bacteria dispersed from the oil into the water. Testers for bacterial content of the dispersed oil present in the water phase should include HAB-, SRB-, APB- and SLYM-. Incubation should be at room temperature and observations made daily or, preferably, every 30minutes using the VBR system.

Consequence: HAB- will commonly trigger as UP reactions in the oil even under conditions that would suggest reductive (anaerobic) conditions exist. DO reactions can occur when there is an excess of anaerobic activity. SRB- will commonly exhibit particulate cycling during some part of incubation. High organic (non- C_xH_y) oils are likely to give the BT reaction with the oils but those with higher sulfate contents are likely to give BB reactions. If the oil is reductive with high organics (non- C_xH_y) then the APB could give DY reactions due to fermentation with fatty acid generation but the acid reaction is likely to be buffered as the fatty acids are utilized by SRB- or CH4- (methane producing bacteria). Generation of reduced organic compounds as black particulates are most likely to be observed in the SLYM- tester as BL reactions following a combination of DS, FO, CP, TH and CL (dominant) reactions.

12.24 Oil, machining

Define: Machining oil are lighter grades of oil used to work metal surfaces during processes such as lathing. Here the oil acts as a lubricant reducing friction heat and rendering more effective precise cuttings. Two problems commonly exist with the use of these oils: (1) oil will enter the atmosphere as micro-droplets; and (2) oils will combine with water to create droplets that can support bacteriological activity. The second problem is the more serious since bacteria can become very active causing degradation of the machine oil leading to losses in efficiency and precision. Additionally these bacteriological activities can also lead to the growth of nuisance bacteria that can cause health problems for the operator and further reduce the efficiency of the equipment due to enhanced corrosion, plugging, and reduced hydraulic efficiencies.

Apply: To investigate the machining oil for bacteriological content the first step is to take a small volume of oil (50ml) and emulsify in a larger volume of sterile distilled water (e.g. 950ml). This oil-water mix needs to be emulsified using a sterile blender until the oil is dispersed in the water. Much of the bacterial biomass would be dispersed by the emulsification process into the water. After 24 hours, extract 90ml of water-oil emulsion for each set of testers being used. Testers for bacterial content of the dispersed oil present in the water phase should include HAB-, FLOR-, APB- and SLYM-. Incubation should be at room temperature and observations made daily or, preferably, every 30minutes using the VBR system.
Consequence: HAB- testers are likely to generate UP reactions relatively quickly in cases where there is significant bacteriological activity. FLOR- testers would detect the activities of significant species of *Pseudomonas* by the generation of the PB or GY reactions later in the incubation. PB reactions are particularly significant since this would indicate a health risk to the operator. In the event the conditions within the equipment are reductive then the generation of the DY reaction by the APB- tester would indicate that a corrosion risk exists. As confirmation of the bacterial activity in the oil then the SLYM- tester should generate CL reactions that might be preceded by DS (indicative of plugging), TH (indicating biofouling) and followed by PB or GY in the event of significant species of *Pseudomonas* being present and active in the oil.

12.25 Oil, tar sand

Define: Tar sands are very reductive localised regimes where generally heavier grades of petroleum hydrocarbons have accumulated within porous media (e.g. sand). These tar sand tend to be exothermic generating higher temperatures than the surrounding geological strata. This heat could have

arisen from the reductive (fermentative) biochemical activities that would be associated with the organics being reduced to the hydrocarbons and possibly even reduced to particulate carbon. Tar sands have the potential to incorporate a diverse and active bacteriological biomass in its synthesis.

Apply: Tar sands are heterogeneous collections of sands and other media incorporating a very reductive organic-rich environment semi-saturated or saturated with water. In the determination of the bacteriological loading in tar sands then the first step needs to be the dispersion of the tar sand so that the active bacterial communities can be evaluated. This can be done by aseptically dispersing 10g of typical tar sand into 100ml of sterile distilled water. Dispersion can employ a sterile blender with homogenisation for long enough to disperse the tar san at room temperature. Once dispersed the 110ml of suspension should be left overnight and then six mid-point 15ml aliquots recovered for BART testing. It is recommended that duplicate testers be employed for the SLYM-, APB- and HAB-. Incubation should be at room temperature and observations made daily or, preferably, every 30minutes using the VBR system.

Consequence: SLYM- testers should initially generate CL reactions that may be preceded by DS but under these reductive types of conditions there should be a terminating BL reaction that will extend from the base slowly to the mid-point of the liquid culture and then move up to under the ball. Because of the organic-rich conditions in the tar sands there should be fermentative activities leading to the DY reaction in the APPB- tester which may, or may not, be buffered. HAB- testers are likely to give DO reactions rather than UP. HAB- testers are also likely to mimic the SLYM- tester and also generate terminal BL reactions.

12.26 Petroleum hydrocarbons, surface leakage & groundwater contamination

Define: Mild steel storage tans are commonly used for the above-ground storage of petroleum hydrocarbons (e.g. gasoline). In the long-term storage one of the major challenges is the formation of pooled water from condensates that then collects under the fuel. Here the water-based pooled environment allows microbiologically influenced corrosion to occur leading to pitting and perforation of the tank floor with leakage of the stored product. This product would infiltrate through the grades down to the static water level where it would collect contaminating the local groundwater. Spillages of petroleum product above grade would also lead to the product collecting at the static water level. Generally this position of the water level below grade becomes a oxidative- reductive interface that would trigger increased bacteriological activity.

Apply: There are three potentially impacted environments when petroleum hydrocarbons enter the below grade environments. These are: (1) semi-saturated formations above the static water level; (2) impacted zone at the static water level where the contaminants have now pooled above the groundwater; and (3) groundwater below the pooling contaminant. Each of these impact sites could offer significant information on the bacteriological nature of the impact. For liquid samples from sites (2) and (3) then the HAB- tester would provide effective evaluation but the methylene blue would have to be pre-dissolved in the cap at the start of the test. For porous samples from all three sites then 1.5g of sample could be dispensed following the procedure discussed in chapter 5.1. Testers suitable for this evaluation would be SRB-, APB-, SLYM- and IRB-. The IRB- would be particularly important if there is a significant iron content (e.g. >5ppm total iron). Incubation would be at

room temperature with daily readings or with the VBR set to time lapses of 30 minutes.

Consequence: For liquid samples the HAB- tester should indicate whether the conditions where oxidative (UP) or reductive (DO) with the time lapse indicating the level of aggressivity. For these samples then the HAB- tester would do the same thing while the SRB- would indicate oxidative activity in highly enriched organic conditions (BT) or reductive high sulfate contents (BB) with the APB- tester indicating reductive fermentative conditions (DY) while the SLYM- tester would normally exhibit the activity by the time lapse to the CL reaction. Reductive organic rich environments would trigger BL reactions in the SLYM- tester that would start in the base and rise rapidly to the half way mark. In the event of an iron rich environment then the IRB- tester will become dominated by brown reactions (such as BG, BC, BR) that are oxidative but, if reductive, then reactions would be dominated by GC, RC, FO, and BL.

12.27 Plug, black

Define: Black slimes and plugs are never viewed as being pleasant. Black is often associated with iron sulfides and hydrogen sulfide (rotten eggs) but it can also be generated by iron carbonates and reduced organic compounds. Essentially these bacteriologically influenced events can cause serious quality and process production issues. Often these form at the oxidative-reductive interface and can cause process failures (whether these are engineered or natural).

Apply: Conditions are most likely to be reductive, possibly with high sulfates and/or organics. To test for significant bacteria in the black plug and sample should be taken aseptically for the investigation. It should be treated as a soil (Table 5.1.1.) and 1.5g of sample used in the testers with 13.5ml of sterile distilled water. The following testers are most likely to yield significant data: SRB- (BB, high sulfate

reductive; BT, high organics potentially oxidative); SLYM-, CL indicate bacterial activity and BL would relate to reductive conditions dominating; IRB-, terminal BL reaction would indicate active iron related bacterial populations functioning under reductive conditions.
Consequence: Black plugs and slime activity would be confirmed by the SRB- tester giving either BT or BB reactions, and the SLYM- and IRB- testers generating a terminal BL reaction.

12.28 Plug, iron-rich

Define: Iron-rich plugs involve bacterial biomass dominated by ferric-forms of iron within a functionally oxidative regime. Over time with growth the biomass accumulates so much ferric-iron that there is a hardening of the growth. Generally the iron ranges from 10% to 95% reflects the maturation of the plug to a point that bacterial activity is minimized. Once hardened the plug significantly impedes hydraulic flow creating a plugging condition.
Apply: Samples for testing should be taken as aseptically as possible and evaluation performed using 1.5g sample with 13.5ml of sterile distilled water using the methods outlined in Table 5.1.1. Recommended testers include IRB-, HAB-, and SLYM- incubated at room temperature. Here the IRB- tester is most likely to give CL, BG, BC and BR in younger samples but move to prolonged time lapses and FO, GC, RC and BL in the matured iron-rich plug. HAB- testers are likely to give UP reactions with extended time lapses in the immature plugs and shift to not detected or DO reactions in the matured plugs possibly with BL if there remains of significant organics in the plug. SLYM- testers will generate CL reactions with time lapses that can be used to assess the maturation state of the plug. For example time lapses of 1 to 2 days would mean a relatively immature plug while lapses of greater than 6 days would mean very small populations associable with a fully matured (spent) iron-rich plug.

Consequence: Maturation of the iron-rich plug is an localised environment varies with location. From the application it is possible, using the SLYM- and the IRB- tester to project the state of maturation of the plug. Longer time lapses would generally mean older with less activity and the occurrences of BL would suggest that there are local reductive environments within the sample.

12.29 Rain, acid

Define: Acid rain has been traditionally linked to the generation of sulfuric acid by sulfur oxidizing bacteria (e.g. *Thiobacillus*) from sulfides. In industrial regions where high-sulfur content fuels have been burnt to generate energy then the stack gases do have a high sulfide content which would become oxidized in the clouds with declining pH in the bio-nucleated water droplets forming the clouds. In some regions where there are high volatile organics being released into the atmosphere there is the potential for these to become accumulated within the bio-nucleated water droplets. Such organics under localised reductive conditions could trigger fermentative activities with the releases of fatty acids that could also cause the pH to decline. There are therefore two likely bacteriological causes of rain: *Thiobacillus* oxidizing sulfides to sulfuric acid; and fermentative bacteria reductively generating fatty acids.
Apply: There is no commercially available BART tester for the sulfur oxidizing bacteria (SOB-) since there has not been confidence in the prototypes developed but the APB- tester can examine rain for the presence of fermentative bacteria. At this time it is only possible to examine total bacteriological populations using the HAB- tester and the APB- tester for reductive types of fermentative bacteria. To do this testing it is recommended that sterile vials be placed out during rain episodes to catch the rain. The sterile outer tube in the field BART testers can be used and will hold up to 70ml of precipitate. 15ml of rainwater samples would be

applied to each tester (HAB- and APB-) following the standard protocol steps. Incubation would be at room temperature and reaction viewed daily or the VBR system set up to operate every 15minutes taking visual images.
Consequence: If acid rain is at least partly the result of fermentative bacterial activities then the pH of the precipitate should be acidic (pH, >5.8). If the acids were at least in part generated by reductive fermentative activities within the clouds then the APB- tester should generate a DY reaction which may be subjected to buffering back to neutral. If this is a significant possibility then the HAB- tester would also have generated a DO reaction confirming that reductive bacterial activities were dominating.

12.30 Rain

Define: Rain relates to the movement of water droplets from bacteriologically influenced bio-nucleation states into free falling water droplets (rain drops). The descending rain will still contain the elements of bio-nucleation which would include the extracellular polymeric substances and viable bacterial cells. This premise considers the rain drop to be a product not of nucleation around “dirt” particles (the classic assumption) but the product of trauma in the living clouds that led to the releases of the rain. If this were to be the case then the rain should still contain many active bacterial flora.
Apply: Ideal circumstances for evaluating the bacterial flora in precipitating rain would be the entrapment of rain within a sterile container during periods of heavy rainfall. Here the deluging rain is descending in large volumes and relatively easy to collect. Aseptic procedures need to be applied to minimise casual contamination of the pooling sample. Of the biotesters it is the bacterial (HAB-) that has been found to function with precision. Incubation can be at room temperature and VBR monitoring is recommended (15 minute time intervals). Generally the HAB- populations range from 1,000,000 to 30,000,000pac/ml.

Consequence: Normally the UP reaction is observed commonly within two days. DO reactions are observed occasionally and this may relate to denser dense or black clouds such as would be dominant in thunderstorms

12.31 Scale, carbonate-rich

Define: Carbonate-rich scales have been considered to be the primary result of chemical processes leading to the precipitation of carbonates. Recent investigations have found that bacteria functioning with bioconcretions are capable of causing this. While the nature of these events remains unclear they appear to occur relatively quickly. It is the IRB- biotester that does respond with the formation of carbonates (see chapter 3.1.2.1 for information on the WB reaction).

Apply: There are two possible scenarios for investigating the formation of carbonates using either the scale that has been aseptically removed; or a liquid sample associated with some scaling event. In the event of scale the method should employ concretion from Table 5.1.1 using the IRB- iron biotester. Incubation would be at room temperature. For liquid samples then 15ml should be added to the regular IRB- biotester and incubated at room temperature.

Consequence: Positive detections of carbonate synthesis within the iron biotester are the development of a white base (WB) within commonly twelve hours (see 3.1.2.1.). Confirmation of the involvement of iron bacteria in the formation of carbonates can be achieved using sterile (autoclaved) samples. If the formation of the carbonates involved bacteria then these sterile controls should remain negative over the test period used.

12.32 Snow

Define: Snow is formed by crystallization of water around extracellular polymeric substances (EPS) that have been generated primarily by bacteria. Generally these flakes form in a circular manner along a flat plain in a manner that appears random. However when snowflakes are synthesised using EPS from pure cultures of bacteria then all of the crystals bear a common pattern. To day snow making equipment often employs cultured EPS to generate uniform types of snow. The challenge with finding the EPS "synthesiser" is to determine whether viable bacterial cells are still present in the snow. There is no certainty that these bacteria will be recovered since the EPS is a product of the biomass; and the viable cells may no longer need to be present.

Apply: Aseptically collect 500ml of snow. Keep the snow under cold conditions (freezer) until ready to attempt to detect the EPS generating bacteria. First allow the snow to melt slowly within a refrigerator to produce approximately 50ml of melt water. Second dispense 15ml of the sample into each of three slime biotesters (SLYM-). Third incubate one tester at 4°C, second at room temperature, and the third at 28°C. Observe daily for eight days for the development of clouding (CL reaction).

Consequence: Testing for the bacterial content in snow is a little "hit and miss" since while the EPS will be there the generating bacteria might not. Three incubation temperatures are recommended since these bacteria (if present) may be strictly cold loving and may grow well at refrigeration temperatures. Contrary to common sense these bacteria may grow quickly at 4°C in the refrigerator. If the bacteria are adaptable then 28°C might give the faster growth. Growth would most likely be observed by clouding which is most easily observed in the VBR tester rack against the lateral black line. If not using the rack then a round black

ball point pen forms a good substitute. Clouding would be seen against the black.

12.33 Tubercle

Define: Tubercles are ferric-rich mounds of biomass. Here the outer layers have the highest concentrations of iron while the central core tends to be rich in biomass. Commonly the tubercle "rests" on surfaces such as steel and there is interaction between the biomass core and the underpinning steel. This commonly leads to pitting forms of corrosion. As the tubercle matures so the ferric-iron content increases and the core biomass becomes concreted. In general the bilk of the bacterial activity lives within the core biomass but is likely to include iron related, sulfide producing, acid producing as well as heterotrophs. This means that the consorm (3 – 15-17 TCL) is complex. To examine the bacteriology there is a need to examine the biomass concretions located inside the core of the tubercle. It also needs to be recognized that as the tubercle ages then so the bacterial activity levels will decline to not detect in a spent tubercle.
Apply: Sampling would be limited to the core biomass in the tubercle only which may be acquired by aseptically removing the ferric-rich outer coating (walls, layers) to expose the core which would appear crystalline, porous and commonly light yellow in color. Remove some of the core material and place in sterile Petri dish. Using Table 5.1.1 concretion analysis apportion 1.0g to each of the following biotesters: IRB-, HAB-, APB-, and SRB-. Use sterile distilled water to make up to 15ml, do not shake, and incubate at room temperature. VBR setting should be to capture an image every 30 minutes or observe daily.
Consequence: For the IRB- iron biotester the first reaction may be either CL (if the tubercle is in an oxidative environment) or FO (if reductive). In the event of the tubercle being associated with ochrous activities then GC or

under more oxidative conditions then BC and BR reactions are likely to dominate. In the event of the tubercle forming in organically richer environments then there is likely to be a terminating BL. For the HAB- biotester the most common reaction would be UP with a possible terminating BL if conditions are more reductive and rich in organics. Under reductive conditions with organics there is likely to be fermentation reflected in the APB- biotester generating DY reactions that may buffer back. SRB- is likely to be present if there is a sulfate rich reductive environment giving a BB reaction. In the event that conditions are more oxidative with higher organics then the BT reaction is possible if the sulfide producers are present. Commonly tubercles tend to be linked to some aspects of corrosion and the APB- and SRB- biotesters when positive would tend to confirm this.

12.34 Water, condensed

Define: Condensed water is water that has moved from a steam (gaseous) phase usually through some form of condenser. This water is not pure water but contains all of the elements in the original water used to make the steam that vaporise at temperatures less than the boiling water. Thus the condensed water will contain chemicals, such as organic volatiles, that have passed with the steam into the condenser. Condensers are essentially hot to cold thermal gradients that differentially trigger the volatiles to return to a liquid state. Thus condensers set up a series of environments along the cooling thermal gradient pathway. If the condenser is functioning continuously then these environments would be relatively stable sites that could support microbiological activity. The type of activity is most likely to relate to biomass that is growing slowly utilizing at least some of the organics that may have travelled with the steam into the condenser. Such growth would do two things: (1) reduce the efficiency of the energy transfer (heat exchange); and (2) impact the quality of the product condensed water.

Apply: The most suitable target group for bacteriologically influenced fouling of the condenser is the HAB- using the bacterial biotester. Aseptic samples of the condensed water should be added as 15ml aliquots into the HAB- tester and prepared following the recommended protocol. Since the water has condensed along a thermal gradient then it is probable that there will be different bacterial communities that had adapted to particular sites along the gradient. To examine the potential for HAB- to have been active at different temperatures it is suggested that duplicate testing at the incubation temperatures of 22°C, 28°C, 37°C, and 55°C. Observations should daily and the VBR system is recommended for at least 22°C with time set for the interval photography at 15minutes.
Consequence: If HAB- is present it is most probable that there will be as UP reactions given that the condenser environment is very oxidative. If the condenser is heavily fouled with biomass then there may be DO reactions indicating these bacteria were growing in more reductive conditions. Detection of large populations of HAB- at 37°C, or 55°C would indicate that the bacteria were active in the warmer parts of the condenser.

12.35 Water, deep oceanic

Define: Water forms the main constituent of the deeper oceans and forms inevitably what appears to be the dominant source of water on Earth. This deep ocean environment is complex built along a seawater salt gradient that is saturated at its deepest points. Microbiologically the ocean is complexed with an upper light zone which is turbulent at the surface. Here phytoplankton is the main synthesisers of the biomass. Below the photic zone, water flows tend to be lateral, slow and constant. Here beyond the zone, where light can still penetrate, there is a permeation of organics and oxygen from above. These chemicals moving downwards trigger activity in the deep scattering layer

(DSL) which is one of the untold marvels of the world. Here, commonly at between 400 and 1,000 meters in the blackness of the void photogenic microorganisms send out pulses of blue light that makes the ocean look like a vast suburbia. When descending below this zone where the "fireflies of the deep" are active then the ocean now exhibit less signs of life in the deep blackness. There are the occasional fish and squid but there are also vast clouds of bio-colloids populated by microorganisms and these persist and even become "slime" columns rising from the ocean floor where the rich sediment beds lay gathering organics and debris as it settles. Seeping out from seafloor are natural gases and petroleum hydrocarbons along with very hot water. These gases and hydrocarbons become a prime feedstock for the deep ocean microflora. The black smokers that occur along the oceanic ridges are particularly striking (12-6) venting black sulfide rich clouds of super-heated water and feeding a rich and diverse microflora which, in turn, provides the feedstock for the animals (typically dominated by crabs and shrimp). This brief introduction shows that the deep ocean environment is extremely diverse and made challenging by the extreme hydrostatic pressures. These pressures are, however, not particularly challenging to the bacteria active within that environment. Consequently it is the salt concentration that is a controlling factor. Temperature declines at depth to stabilize at around $4^{o}C$ and so this is a natural incubation temperature which coincides with that of refrigerators. There are therefore many different, and challenging, environments but this protocol will simply use HAB- testers with incubation at$4^{o}C$ and the main variable would be salt concentration.

Apply: There are two biotesters that would detect the general bacteria active degrading organics in the deep ocean environment. These are the HAB- and SLYM- testers. Critical in the testing is the fact that all samples would employ 15ml of sample to assure that the salt concentration does not change and remains compatible to the local

microflora. Incubation would be at 4^oC which would be compliant with the environment from which the sample was taken. HAB- testers would need to have the methylene blue pre-dissolved in the cap (see 3.4.1 for details). Failure to pre-dissolve would cause the methylene blue to turn green (4 to 8% salt) and be completely unusable at high salt levels. Commonly it would be expected that UP reactions would commonly occur given the oceanic environment is oxidative but if samples are taken from within bio-colloids then DO reactions may occur. SLYM- testers are likely to give CL reactions followed by BL if the sample is from a bio-colloid and more reductive. In the event that the sample is from an oxidative colloidal biomass then TH, DS or CP may also be generated in the SLYM- tester.
Consequence: In sequential vertical profile sampling of the deep ocean it can be expected to see radical shifts in the bacterial populations relating to activity zones that are created by the phytoplankton, DSL, biocolloids, sea floor sediments and active seeps. It is therefore unlikely that there would be an homogeneous population but rather local variations reflecting those environments.

12.36 Water, produced from gas well

Define: Natural gas wells are perceived to be not suitable environments for bacterial activities. This would be based on the notion that bacteria could not grow in gas (e.g. methane). However there is always groundwater associated with the gas well either at a distance from the well, close to the producing zone, or inside the borehole and coming through the perforations. This groundwater can provide a suitable environment for bacterial activities which then appear in the produced water recovered with the gas. If there were significant populations of bacteria in produced water from gas wells that could be related to: (1) upstream biofouling of the gas well leading to plugging and corrosion

issues; (2) the produced water acting as a conveyor system for any methane producing bacteria that might be significant contributors to the natural gas reserves; and (3) bacteria actually causing biofouling problems in the equipment associated with the gas well itself.

Apply: The most suitable biotester is the HAB- tester. For this test regular 15ml samples of the produced water (depressurized if collected under pressure) will work well in the HAB- tester. There is however one concern that there could be interferences from volatile petroleum hydrocarbons in the sample. When present these volatiles will react with the dissolving of the methylene blue. To prevent this then the methylene blue indicator (dried in the cap of the tester) needs to be pre-dissolved in 1ml of sterile distilled water prior to the start of the test. After one minute then the solution in the cap can be emptied into the tester to which 14ml of produced water sample has been added. Do not shake the tester but allow free diffusion of the methylene blue into the culturing fluids. Incubate at room temperature with daily observations for any reactions; or use the VBR system set to incubate at 28°C.

Consequence: Produced water can have a very high HAB- population often measurable in the millions of pac/ml. UP reactions indicate oxidative conditions exist. In the deeper confines of gas wells such oxidative conditions would seem unlikely but these conditions can be achieved through the use of electro-magnetic forces to protect the casing (cathodic impression) and drive the down-hole pumps and equipment. Here such forces could then cause the electrolysis of some of the produced water generating oxygen and hydrogen. DO reactions would indicate that the conditions in the produced water were reductive and there is under these conditions a lower probability that the natural gases and volatile hydrocarbons are being effectively degraded. Instead in the DO reaction the bacterial activities may relate to fermentative activities utilizing other naturally

occurring organics in the groundwater and formation materials.

12.37 Water, produced from oil well

Define: Crude oil being extracted from oil wells is likely to be a mixture of oil and water with the water content rising as the well appears to be becoming exhausted. Here the production of crude will decline while the production of water increases. This shift can be related to the interaction between the microorganisms in the crude and the water. Commonly the water is mined from the crude and bound within biomass associated with the crude. Some times this mining of water leads to the generation of "black goo" (see 12.4) when the water is effectively bound into the biomass. Once the water reaches higher concentrations then the water will pool beneath the crude as produced water. Such water produced and separated from crude is likely to have similar characteristics to the water from gas wells (see 12.38) but with a greater diversity in organic feedstock including the full spectrum of petroleum hydrocarbons. An additional factor that could be significant is the potential for the generated biomass to contribute to the plugging of the crude flow lines to the perforations. In this event then there may be very high bacterial populations in the produced water with very low flows of crude.
Apply: The same methodology using the HAB- biotester can be applied as described for 12.38. However there is a greater risk of secondary corrosion resulting from the bacterial activities and the SRB- and APB- testers using 15ml of produced water could be employed.
Consequence: For the HAB- biotester similar conclusions could be expected as in 12.38 except that there is a high probability that there would be a terminal BL reaction

occurring relatively quickly after the initiating UP or DO reaction. For the SRB- the results would be of concern if either the BT or BB reactions were observed because of the association of SRB- with pitting or perforation forms of corrosion. BB reactions indicate a greater potential for deeply set pit corrosion likely to lead to perforation. BT reactions are more likely to be associated with lateral types of more extensive pitting which would weaken the steel and lead to increases in porosity as micro-perforations occur. APB- generates the DY reaction which would indicate a potential for a slow form of lateral pit corrosion.

12.38 Water, saline

Define: Water moves through a cycle from relatively pure (in rain and snow) to saline as the water moves from shallow to deep in surface waters and also then migrates down through groundwater formations. Thus there is a vertical profile with salt concentrations rising with depth in both the deep oceans and crust. Occasional these salt-rich waters seep up into the surface environment where the salt becomes toxic for the plant and animal life. For the bacteria, there is generally a greater tolerance to salt partly because of the protective functions in the extracellular polymeric substances which keep the salt away from the cell walls. Generally salt tolerance amongst bacteria can be grouped: (1) salt sensitive, cannot tolerate more than 0.01% salt; (2) moderate salt tolerant, generally function within the range of 0.01 and 8.0% with most bacteria becoming salt limited at 2.0 to 4.0% salt; (3) high salt tolerance, usually function in the range of 4 to 12% salt; and (4) salt dependent, cannot function with less than 12% but do function in limited ranges between 12% to saturation. In saline waters the natural salt concentration remains when 15ml of sample is applied to the biotester. If dilution is required for a solid, semi-solid or porous sample then the salinity of the sterile diluant becomes critical. For the various salt ranges

discussed above then the optimal salt concentration (using seawater salt would be: (1) salt sensitive, use distilled water; (2) moderate salt tolerance, 0.8% salt; (3) high salt tolerance, 8% salt; and (4) salt dependents, 14% salt.
Apply: In the examination of the bacteriological content of these samples may use those biotesters that may be applicable for the perceived bacteriological problem with the fall back position being to use SLYM- biotesters since these have a high sensitivity to a wide range of bacteria with minimal concerns. **Consequence:** This set of procedures is particularly relevant to the high tolerant and dependent salt groups (3 and 4) and may not be so significant in the low salt tolerant groups (1) and (2).

12.39 Wastewater, sanitary

Define: Sanitary wastewater treatment is bacteriologically one of the most interesting phenomena since the bacterial consorms coming into the system are dilutions of the intestinal flora. These organisms have entered an alien environment at a lower temperature and they go into trauma. Wastewater treatment plants (WWTP) are designed to degrade all of the organics in the wastewater along with eliminating the risk from any pathogens present. Within WWTP the prime focus is on the safe discharge of the treated wastewater. In simple terms this means removing the solids physico-chemically and then degrading the organics bacteriologically. The final discharge into the environment has to have an acceptably low oxygen demand and be free from harmful chemicals and potential pathogens. The primary objective is to reduce the biochemical oxygen demand (created primarily by bacteria respiring / breaking down the organics) and eliminate the health risk by effective removal of any potential pathogens in the wastewater. This protocol addresses the ability of the WWTP to reduce the

active bacterial loadings from the very high levels seen in the primary influent (PI) to acceptably low levels in the final effluent (FE).

Apply: Protocol for this evaluation of the percentile reduction from PI to FE is performed using the HAB-biotester that has to use the VBR system for precision. Here triplicate samples of the PI and FE are set up in the VBR following the standard recommended protocol. Generally the test is complete in 18 hours using the incubated (28°C) VBR system with the timed intervals for recording images set at five minutes. This system allows the archiving of observed reactions and time lapse that are then converted to predicted populations as pac/ml. Percentage reduction in population (PRP) from the PI to the FE is performed using the average (mean) value from the triplicated tests. Here the equation is:

$$PRP = 1 - (FE / PI)$$

Where FE is the mean population (pac/ml) for the three FE samples tested and PI is the mean population (pac/ml) for the three FE samples tested.

Consequence: The efficiency of the WWTP is reflected in the PRP generated. An ideal would the achievement of three orders of magnitude reduction in the PI population by 99.9%. From the present studies it would appear that 99.7% is a more realistic goal and the efficiency would be dependent upon the type of treatment process being applied.

Chapter 13

Risk Assessment for Corrosion, Plugging and Health

13.1 Using the BART Analytical system for the projection of Risk.

There are three recognized risks in water that can be estimated on the basis of the reactions observed during BART testing. The analytical system was qualitative in that it projects risk on the basis of the types of reactions that are observed for the BART testers when applied to a sample. There are three major risks evaluated: corrosion (C), plugging (P) and health (H). These are calculated individually from zero to nine. In BART-SOFT version 6 the risks are only presented from zero to six because all of the five major categories (negligible, minor, medium, major and extreme risks) do occur within the zero to six scale. All risk values between 6 to 9 are considered extreme and not include in the BART-SOFT version 6 except as 6 (meaning >6 and extreme).

Corrosion relates to microbiologically influenced activities that cause damage to metal or concrete structures. This usually begins with compromises to the surfaces where the microbes are active and then moves into the structures. These compromises lead to lateral dishing, pitting and eventually full perforation of the metal or concrete structures. There is also an ongoing loss in strength as the metals or concretes corrode.

Plugging is a product of the growth of biomass (from biofilms) that then begins to occupy significant volume

displacing the resident liquids or gases. This biomass can form into slimes (commonly seen in cooling towers and heat exchangers) or as growths within porous media (e.g. sands, gravels), or within fractured rock. Plugging can also affect hydraulic flows not just for water but also for oils. This is because some of the biomass extends into the flowing fluid and forms bio-colloidal structures that resemble slime webs. Biomass can also cause plugging within various grades of crude oil when the biomass begins to bind the water into defined structures often with coatings of asphaltenes. Gas flows (e.g. natural gas, methane) can also be affected by the biomass plugging in the formation materials or actually around the perforations of the well itself. Here the impact of the biomass in binding water closer to the well would initially cause diversionary flow but then it would diminish gas flowing to the well. In water wells the most commonly "observed" plugging is in the screened slots or perforations in the well casing. Video logging the well would commonly show when plugging is occurring and often which zones are the most affected.

Health risks relate to humans or farmed animals that are using the water as a source for drinking. Traditionally health has been defined using the presence (bad) or absence (good) of coliform bacteria. Coliform are more commonly associated with enteric infections and are detected as either total coliforms (broad sweep), fecal coliforms (narrow sweep), or as *E. coli* (specific health risk). The health risk generated here is a broader assessment of risk to humans and animals from the various bacteria that might be present in the water. This risk includes some of the opportunistic bacteria that can be active in the environment but can cause disease in humans or animals (nosocomial pathogen).

13.2 Confidence rating

Risk assessment is always given on a scale from zero (no detected risk) to nine (significant probability of risk. The highest value for the risk predictions is when all five BART types of tester are employed. When less than the full number of BART testers are used then the value of the risk assessment is reduced corrosion risk (CR), plugging risk (PR), and health risk (HR) may be calculated if the IRB-, SRB-, HAB-, SLYM-, and DN-BART testers have been used. If less than the five BART testers are used then risk analysis will lose its precision. Note testers that are negative when included in the test are included by default but do not contribute to a positive risk assessment.

13.3 Corrosion risk (CR)

Corrosion risk is generated by all five testers and the reaction relevant to corrosion are show as “weight C” shown from 0 to 9 with an ascending degree of risk. Relevant corrosion by C weight are given separately for each tester group: IRB- (13.1), SRB- (13.2), HAB- (13.3), SLYM- (13.4) and DN- (13.5). In this assessment the major corrosion risk is primarily based on the SRB-BART which would deliver a C weight of 9 if BB observed and 3 if a BT is observed. Generally BB reactions occur where there is radical pitting and perforation particularly of steels. It should be noted that a recent addition to the BART testers is the one for acid producing bacteria (APB-). This tester will detect fermentative activities that could lead to the pH dropping into the 3 to 5 range which can also create corrosive conditions particularly under conditions where high organic loadings exist under reductive (anaerobic) conditions.

Table 13.1 Corrosion risk, IRB- BART

	RX	weight C
IRB	BC	1
	BG	2
	BL	1
	BR	0
	CL	1
	FO	3
	GC	0
	RC	0

Corrosion risk is equal to the sum of all corrosion reactions bearing a significant C weighting (1 or higher). The risk is calculated as the sum of all positive C weighted reactions observed for all five tester types divided by the number of reactions observed. Risk for corrosion can therefore be 9 (severe risk), 3 (moderate risk), 1 or 2 (minor risk exists. See 13.6 for more interpretation.

Table 13.2 Corrosion risk, SRB- BART

	RX	weight C
SRB	BT	3
	BB	9

Table 13.3 Corrosion risk, HAB- BART

	RX	weight C
HAB	UP	0
	DO	2

Table 13.4 Corrosion risk, SLYM- BART

	RX	weight C
SLYM	DS	1
	SR	0
	CP	0
	CL	1
	BL	1
	PB	0
	GY	1

Table 13.5 Corrosion risk, DN- BART

	RX	weight C
DN	FO	0

13.4 Plugging risk (PR)

Plugging is primarily an expression (as P weight) for evaluating the manner in which the biomass generated by the biofouling now interferes with liquid or gaseous flows through porous media or over surfaces. Net effects of biomass plugging would be restricted flows in the designed production, greater generation of back-pressures and smaller yields. Generally these types of activities are step-wise through periods of stability to sudden dramatic changes (e.g. drops in flow) in a manner that is repeated in consistent manners. . Relevant corrosion by P weight are given separately for each tester group: IRB- (13.6), SRB- (13.7), HAB- (13.8), SLYM- (13.9) and DN- (13.10). Plugging risk is the assessment of all reactions generating a positive P weighting.

Note that the maximum value that can be ascribed is 9 and the minimum value of 0 only in the case of no plugging risks being observed. All fractions of the PR would be rounded upwards.

Table 13.6 Plugging risk, IRB- BART

	RX	weight P
IRB	BC	4
	BG	6
	BL	6
	BR	7
	CL	2
	FO	1
	GC	3
	RC	0

Table 13.7 Plugging risk, SRB- BART

	RX	weight P
SRB	BT	0
	BB	0

Table 13.8 Plugging risk, HAB- BART

	RX	weight P
HAB	UP	4
	DO	2

Table 13.9 Plugging risk, SLYM- BART

	RX	weight P
SLYM	DS	3
	SR	5
	CP	0
	CL	3
	BL	4
	PB	0
	GY	3

Table 13.10 Plugging risk, DN- BART

	RX	weight P
DN	FO	3

13.5 Health risk (HR)

Health Risk (when it occurs) is serious and generates a number in the scale of 3 to 9 and is based on a summation of all of the recognized reactions with 9 being the highest number for the HR. There is no averaging or allowances for the number of reactions. Hence a HR of 3 would only be reported is GY was the only recognized reaction. In the case of multiple reactions then it is most likely that the risk would be 7, 8 or 9. There are only two BART tester types that are employed in the HR calculation: IRB- BART, Table 13.11; and SLYM- BART, Table 13.12. The only reactions included are those that are most likely to present a health risk resulting from the samples tending to be higher in assimilable organic content and reductive. Where the HR is found to be significant (see 13.6) then confirmation should be undertaken using the total coliform test.

Table 13.11 Health risk, IRB- BART

	RX	weight H
IRB	BL	9
	GC	5
	RC	5

Table 13.12 Health risk, SLYM- BART

	RX	weight H
SLYM	BL	9
	PB	7
	GY	3

13.6 General CPH risk interpretation

Calculation of the CR, PR, and HR risks involves taking all reactions that were observed and designated a weighting (e.g. weight C, P or H). Risk for all three is defined by a count of the number of reactions observed that did generate a weight (Rw) and the sum of the weights obtained (Sw). Calculation of the risk (R) is generated by averaging the recognized weighting given to the risk (Rw) by the count of reactions that were recognized (Sw). The equation therefore is:

$$R = Rw / Sw$$

Highest possible weights, counts and maximum risk values are given in Table 13. 13.

Table 13.13 Summary calculation of highest reaction numbers, potential incidents and average risk values.

	Highest Rw	Highest Sw	Average R
CR	26	15	1.7
PR	53	18	2.9
HR	38	6	6.3

Note: Highest Rw is the sum of all possible reactions recognized as having a value more than 0 in the evaluation; highest Sw is the sum of the reaction incidents that can maximally occur; and average R is the value achieved if all possible reactions were to be detected. Average R therefore represents the mean values that would translate into a significant risk for CR, PR, and HR.

Calculation of risk is dependent upon the number of recognized reactions for the CR, PR or HR coupled with the number of incidents that have occurred. In all three risk analyses the maximum value that can be generated is 9 when the only reactions recognized have a value of nine. For example an CR of nine would mean that the only reaction was a BB reaction on the SRB- BART tester. For PR the only time that maximum would be generated would if seven was generated by the BR reaction in the IRB- BART tester. For HR then the maximum risk would be achieved if one or both of the BL reactions go positive for the SLYM- and or IRB- BART testers. The prediction of risk using the CR, PR or HR methods is subject to shifting depending upon which reactions are detected in calculating the final risk. Because of this shifting then the final risk projection is semi-qualitative ranging through no risk (0), to low risk, through medium, high and extreme risks depending upon the spectrum of activity observed. Because of this the five risk categories change with the three risk factors (CR, PR and HR) and are dealt with separately below in Tables 13-14, 13-15, and 13-16. Each table incorporates the risk levels (left column) from extreme down to negligible followed in the idle column by calculated risk factor generated by BART- SOFT version 6 on a scale from 0 to 9. In the right hand column the risk factor is shown by color with purple (extreme risk), red (high risk), yellow (moderate risk), pale green (low risk) and green (negligible risk).

13.7 CR Risk Interpretations

Effectively the corrosion risk operates over a practical scale from 1.75 to 3.75 with higher values representing an extreme risk.

Table 13.14 Corrosion Risk (CR) Projection

Severity of Corrosion Risk	CR Computed Risk	
Extreme	3.75 to 9.0	
High	2.8 to 3.7	
Moderate	2.25 to 2.75	
Low	1.75 to 2.24	
Negligible	<1.75	

13.8 PR Risk Interpretations

Effectively the plugging risk operates over a practical scale from 2.3 to 5.0 with higher values representing an extreme risk.

Table 13.15 Plugging Risk (PR) Projections

Severity of Plugging Risk	PR Computed Risk	
Extreme	5.0 to 9.0	
High	4.0 to 4.9	
Moderate	3.2 to 3.9	
Low	2.3 to 3.1	
Negligible	<2.3	

13.9 HR Risk Interpretations

Effectively the health risk operates over a practical scale from 1.75 to 3.75 with higher values representing an extreme risk.

Table 13.16 Health Risk (HR) Projections

Severity of Health Risk	HR Computed Risk	
Extreme	3.75 to 9.0	
High	2.8 to 3.7	
Moderate	2.25 to 2.75	
Low	1.75 to 2.24	
Negligible	<1.75	

Further Information

This book "*Standard Methods for BART Testing in Environmental Investigations of Microbiological Activities*" is formed as a scientific opinion based upon working in the sphere of microbiological ecology since 1959. As a result these standard methods are based not upon established scientific literature but upon the practical forensic investigations. These investigations led to the development of the patented biological activity reaction test (BART) which has been commercially available since 1990. This BART product has been used primarily by engineers and consultants in the water, chemical, oil and gas industries where it has proven to be simple to apply while being sensitive to bacterial activities and precise. Over the last twenty years, there has been a steady stream of feed back from users. In consequence of this steady feed back, the BART testers have been improved and received ISO 9001-2000 certification in 2001. Manufacture of the testers has been solely performed by Droycon Bioconcepts Inc (Regina, Canada).

There are references to work performed using the BART testers and these have been summarized in a series of books published since 1993 and covering various applications with the primary focus on groundwaters, biofouling and corrosion. These books are listed below with (bracketed) the major focus in each publication:

Cullimore, D. R, (editor, 1987) International Symposium on Biofouled Aquifers: Prevention and Restoration. American Water Resources Association Technical Publication Series TPS-87-1, published by AWRC, Bethesda, Maryland (this was one of the first attempts to scientifically address the issue of biofouling in groundwaters and the methods for regenerating impacted water wells).

Cullimore, D.R. (1993) Practical Manual of Groundwater Microbiology. Lewis Publishing, Chelsea, Michigan, pp. 403. (this became a bestseller for the publishers and it introduced the BART testers as major microbiological forensic tools. Copies of this book are available free of charge from a number of websites)

Cullimore, Roy (1999) Microbiology of Well Biofouling, published by Lewis Publishers / CRC Press, Boca Raton, Florida. 435pages. (this book refined the methodologies that had been developed from 1986 to 1999 for the application of the BART testers primarily in water environments).

Alford, G. and Cullimore, D. R. (1999) The Applications of Heat and Chemicals in the Control of Biofouling Events in Wells, Monogram in the Sustainable Water Well Series (D. R. Cullimore, series editor) published by Lewis Publisher / CRC Press, Boca Raton, Florida 205 pages. (From 1988 through to 1999 there was a series of investigations of the ways in which BART testers could be used aid in the regeneration of biofouled wells in the USA. Many of these projects were developed with the support of the U.S. Army Corp of Engineers.)

Cullimore, D.R. (2000) Practical Atlas for Bacterial Identification published by Lewis Publishers / CRC Press, Boca Raton, Florida. (From 1966 there was seen to be a need to develop a more condensed approach to the classification of bacteria. This book which was a best seller introduced two dimensional mapping and the BART technologies to the classification of bacteria).

Church, R. Warren, D. Cullimore, R. Johnston, L. Schroeder, W. Patterson, W. Shirley, T. Kilgour, M. Morris, N. and J. Moore (2007) A study of Living History, World War II Shipwrecks in the Gulf of Mexico. Artificial Reef

Effect in Deep Water, U.S. Department of the Interior, Mineral Management Services, Gulf of Mexico OCS Region, New Orleans. 239 pp. published by PAST Foundation, Columbus, Ohio. (This book deals more with Deep Ocean and WWII steel shipwrecks but does touch on the microbiology and the BART testers. This book (ISBN978-0-9799909-3-9) should be in all school libraries as an introduction of Deep Ocean life and corrosion).

Cullimore, D. Roy. (2008) Practical Manual of Groundwater Microbiology, second edition. Lewis Publishing, Chelsea, Michigan, pp. 376. (From 1999 to 2008 there were a number of significant developments in the understanding of groundwater. The second edition was therefore published to improve the understanding of biofouling in groundwaters and how the BART testers could be used).

Cullimore, D. Roy. (2010) Practical Atlas for Bacterial Identification, second edition, published by CRC Press, Boca Raton, Florida, 327pp. (This book represented a major departure from the traditional Linnaean approaches developed as early as 1830. Here the premise is that all bacteria primarily function in community structures and their identification involves, in some case, the application of BART testers. This second edition should be appealing to those that have to contend with microbiological challenges in various environments).

There are numerous reports that have used the BART testers. Below is one of the longer assessments of water well rehabilitation techniques:

Jeffrey B. Lennox, CPG of Leggette, Brashears & Graham, Inc (2007) AwwaRf project 464, “Application of Well Condition Assessment and Rehabilitation Techniques.” Published by AWWA Research Foundation, Denver, CO. (This document was a detailed assessment of the various common practices

applied in the groundwater industry for the rehabilitation of biofouled water wells mainly in eastern USA. BART testers were used through out to indicate the potential levels of bacterial challenges and effective regeneration of the treated wells).

The BART testers run under the patent listed below but has also been the subject of further patent applications and trademarks:

Cullimore, D.R. and Alford, G.A. (1990) Method and Apparatus Producing Analytic Culture, U.S. Patent number 4,906,566 (Concepts were developed in 1986 that was accepted in 1990)

Individual reactions and interpretations were developed as the different testers were developed. Reactions were originally categorized numerically (1993) since there was some confusion over the color recognition particularly by observers have more limited color recognition. The movement to two letter coding was introduced to define reactions in 1999 and color comparisons were introduced using the standard Pantone® colors in 2010. Once major challenge with the colors is that every individual test that is considered positive may have slightly different shades of color.

Each of the reactions in the BART testers has some relationship to physico-chemical processes that occur in connection with positive (bacteriologically active) tester. All of these reactions are well founded in chemistry with the exception of the formation of visible growths (as clouding, slimes, threads, floating plates and colloids). These are primarily the result of the growth of biomass within the

tester either as dispersed or flocculated biocolloids, as defined structures such as sheaths (tubes) or floating plates.

Other reactions commonly represent a direct and indirect color shift resulting from interaction between the active bacteria and either the chemistry of the tester, or the chemistry of the sample. Essentially the chemistry in the tester is defined but the chemistry in the sample cannot be defined since it is dependent upon the environment from which the sample was taken. While reactions are established under defined conditions when natural samples are applied then these reactions can be affected by the chemistry of the sample. This means that the nature of the sample can affect the interpretation of the reaction by shifting the color spectrum being observed. This needs to be taken into account when interpreting reactions. These are described in the next paragraphs.

Iron in samples used in testers is possibly one of the major interference factors. This is because bacteria in the sample can shift iron relatively easily from the oxidized insoluble forms (ferric) dominated by yellow, red, brown color shifts; or to reduced soluble forms (ferrous) which usually are in the green color shifts. Iron-rich samples are most likely to interfere with reaction patterns particularly when the total Fe exceeds 3ppm. This does not necessarily render the reaction unreadable but does mean that there will be minor color variations. Examples of these interferences for different testers are described below:

IRB- tester is affected by iron content in the sample and this is addressed during the four phased interpretation (see 3.1.2). If there are iron oxidizing or reducing bacteria in the sample along with a significant iron content then the first phase reaction will reflect this with GD (green diffusion) in the event of iron reduction dominating; and YD (yellow diffusion) dominating in the event of iron oxidizing bacterial

activities occurring. Usually these two reactions are not taken as positive declarations for the IRB- tester but they do indicate the type of events that are likely to occur in phase two. Generally these two (GD and YD) reactions occur without clouding and are not considered as being directly linked to bacterial biomass generation. They should be view as precursors to second, third and fourth phases of the reactions. GD will usually lead to FO and on some occasions: GC, RC and BL whilst YD would move towards CL, BG, BC and BR and possibly a BL. It is when any of these secondary or tertiary phased reactions first occur then the tester is declared positive. It should be noted that while the FO reaction is a signal for reductive activities secondary reactions can involve iron oxidizing bacteria. Here the foam forms a ring around the ball and iron oxidizing bacteria can then grow on top of, or in the foam generating the typical ferric colors. It is not uncommon to see the foam generate orange to brown edges to the bubbles as the ferric forms from the activities of the iron oxidizing bacteria. On rare occasions the ferric product will form as an encrustation over the foam but penetrate into it.

For the IRB- tester there can be additional reactions where iron is significantly present in the sample. These relate to cases where there is a terminal black liquid (BL) forming as a tertiary reaction. This most commonly would involve the generation of black iron sulfides but it could also involve the generation of black iron carbonates (siderites). This is more likely to occur if there is an abundance of carbonates in the sample (generating a primary WB reaction).

Iron can also interfere with the HAB- tester in a minor manner. Here the iron from the sample reacts to create a yellow instead of clear solution when the methylene blue begins to bleach out (become reductive). In the SRB- tester there is already an abundance of iron from the tester's pellet. This interacts with the hydrogen sulfide to form black iron

sulfides. In the event that there is an excessively high level of iron in the sample then the culturing sample may generate yellow, orange or light brown colors. This is, however, not common. There has not been evidence of the iron content interfering significantly with the other testers.

One reaction that has caused particular concern is the BL (black liquid) reaction that occurs most commonly in the IRB- and SLYM- testers. Originally it was considered that this was caused by the generation of daughter products around iron sulfides or carbonates. However more recent researches into the BL reaction phenomenon have found that the BL reaction can occur in samples with low iron content. Here the BL forms as an ascending formation of fine black particulates that remain in suspension while forming from the base upwards. This reaction is particularly common in the SLYM- and HAB- testers. From analysis of the BL-positive testers it would appear that the black particulate suspensions triggered by reduced organic carbon compounds including elemental carbon. Conditions in these testers can become extremely reductive in the base that could trigger the stripping down of organics to hydrocarbons and carbon. Frequently these types of BL reactions also lead to the generation of gases which evolve from the tester. Analysis of entrapped gases has revealed the presence of methane and hydrogen, both of these can be the result of reductive biochemical activities involving the bacteria active in the tester.

Turbid (cloudy) water samples have been a perennial problem when attempting BART tests since they make it much more difficult to determine the points when a reaction would be declared. This has been addressed in 4.16. Here the only solution is to dilute the sample with sterile distilled. In the event that the sample ahs a high salt content (see 4.15) then the better diluant would be filtered (0.45microns) and sterilized sample. This would mean that the bacteria would

not be traumatized by shifts in the salt concentration. Generally where dilutions are necessary then the normal range is between one and three orders of magnitude. This would mean that the time lapses would be lengthened with the greater dilution. However the predicted population could then be corrected by multiplying the number by the dilution factor. For example testing performed at two orders of magnitude dilution would mean the predicted population would have to be multiplied two orders of magnitude (i.e. x100).

Protocols are defined by the sample and the "*Certificate of Analysis*" included with each box of testers gives the standard method for water samples. This standard methods book includes protocols modified for soils, turbid waters and other environments. Chapter twelve specifically addresses some of the more unfamiliar environments where limited investigations have been undertaken. In the two editions of the "*Practical Atlas for Bacterial Identification*" some references are made to these environments. The second edition includes a partially tester-based identification of the bacterial consorms dominating in these habitats. Essentially the whole process of recognizing bacteriologically influenced activities shifts from the Linnaean concepts (designed for plants and animals) to a more holistic approach. The bottom line is to know what the bacterial communities are doing within their habitats to influence the local and regional environments.

IN MEMORY

GEORGE ALFORD

George Alford lived in Daytona Beach, Florida, died in 2009 after battling acute leukemia. He will be remembered by many in the ground water and environmental fields as “Big George”. He instinctively knew the ways of Nature and how to read the signs. Rejected formal education as being simply boring and yet he came to be one of the most respected people with a unique knowledge of water wells and the things microbes did to them. Basically he saw Nature as just healing the intrusion made by the wells into the groundwater. George developed many techniques to fend off microbial plugging of wells. He tried gamma radiation at a time when nobody though it practicable. After many trials and successes it ended with the patented blended use of chemicals and heat treatment called BCHT. This technology worked and made water wells sustainable provided the proper monitoring and maintenance was done. George dreamed of one day of having a camera pointed at the ground that would take pictures of the microbes plugging deep down water wells around the bore hole and screens. That hasn’t happened yet but one day that dream will also come true. George will not be forgotten by those who crossed his path for he was larger than life, always prepared to listen and loved to find the solution. We worked together since 1981 when George phoned me up to tell me that you can’t heat up water wells. After the phone went down with George thinking I was crazy he wheeled an old water heater over one of his plugged wells and flushed

it with hot water. It worked, the microbes died, the well thrived again and George phoned me back to say yes you can treat wells with heat. But that was just the beginning of a long journey with failures and successes and George loved to learn from the failures but never crowed about successes. George would simply hum, say “soon come, man!” and see how he could make the treatment even better.

Today there is a little piece of George in the memories and hearts of the many he talked to, the few he argued with, and the ones who simply said well George was right!

Times well spent and memories now shared

Thanks George

Roy Cullimore

2 March 2010

Foreword

In some ways George Alford was a big part of looking at ways to detect these nuisance bacteria that can grow in water and he thought there needs to be a simple test that anyone can use. Well I helped him with BCHT and he helped me with that simple test. This Standard Methods of BART testing has its foundations, indeed deep roots, into those conversations that gradually by trial and error to the biological activity reaction test (BART™, Droycon Bioconcepts Inc, Regina, Canada). The first BART tester was made in 1986; the first sale was in 1990 with the beginning of manufacture. There has not been an attempt to grab the market but rather just let the sales grow (organically). This slow growth has been a Godsend since we have been hearing back from customers, making corrections, improving the product. Today the BART tester is made in-house using plastic injection moulding, clean rooms and, since 2001 full ISO 9001:2000 certification to make sure we are doing it right.

This book "*Standard Methods for the Application of BART testers in Environmental Investigations.*" has been produced to standardize the methods. In a typical week I get three or four e mails and a similar number of telephone calls enquiring about the applicability of the testers as they have become adopted. In general the BART testers are seen to be simple to use in the field or the lab, gives precision, without undue need for math and chemistry. Chapter 11 addresses the potential use of BART testers to unlock bacterial activities in places where no agar plate has dared to explore. These testers have been successful because

they are both simple to use (for the operator) and comfortable for the bacteria whose activities are being investigated.

There are many who now guide the direction of the BART testers to achieve their goals of detecting specific bacteria with precision and speed. In Droycon Bioconcepts we have a team of people who all share their enthusiasm to make the testers better. I would like to thank particularly: Natalie, ("and where's the money coming from for this?" Vincent (Webdude extraordinaire), Derek ("tweak, tweak!"), Myrna ("the inventory is only this big"), Donna ("it's really shiny now"), Jason ("But Dad!"), Ahmed ("yes it will be done by 4:30!"), Behrooz ("why don't you?"), Kristina ("next month India and then I might go to Nepal!"), and Lori ("have they phoned yet?"). Thank you all for making a team effort that works.

Beyond the horizon of Droycon there are the many without whose help, thoughts, questions, suggestions and criticism we could not be anywhere near where we are today. The list would go on for pages but I will just say thanks you guys and keep it coming. Finally I would like to acknowledge the Prokaryotes for allowing me to play with them and maybe understand them a little better. Silence was the beginning and a void will be the end but we must all remember that, while dwelling on Oceania (also known wrongly as the Earth), the biggest all-embracing biomass is formed by those Prokaryotes who just do what they do best.

D. Roy Cullimore
14 June 2010

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