ENVIRONMENTAL TECHNOLOGY VERIFICATION

OF THE

IRB-BART™ TESTER

FOR THE

DETECTION AND EVALUATION

OF

IRON BACTERIA IN WATER

AT THE SEMI-QUANTITATIVE AND SEMI-QUALITATIVE LEVELS OF PRECISION

Droycon Bioconcepts Inc., 315 Dewdney Avenue
Regina, Saskatchewan, Canada S4N 0E7
(306) 585 1868
(306) 585 3000
www.dbi.ca
DRC@DBI.sk.ca

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Tables

1, Introduction

This document relates to the verification by ETV Canada Inc of the IRB-BART™. The name of the trademarked product (IRB-BART) stands for biological activity reaction test and, for the purposes of verification, is limited to the iron related bacteria (IRB) that cause in any manner nuisance bacteriological activities that can, on occasions, be associated with water. These testers are exclusively manufactured by Droycon Bioconcepts Inc. (DBI), Regina, Saskatchewan. As a part of the quality management objectives DBI has obtained ISO 9001:2000 registration in August, 2001. The verification of the IRB-BART tester has been based on a scientific and technical basis in which both internal and independent information was presented. In addition this document also addresses the relative convenience and confidence of this test in comparison with existing laboratory and field tests at the semi-quantitative and semi-qualitative levels of precision.

2 Concepts

The concept involved in the IRB-BART tester is presented in seven parts. These may be subdivided into the historical development and selection of an instrument that would detect IRB nuisance bacteria, the level of precision that can be achieved and the quality management processes applied in the manufacture and verification.

2.1 Historical

During the nineteenth century, there was a growing acceptance of the ubiquity of microbial activity and the realization of the impacts that these microorganisms did have directly and indirectly on human society. In the latter part of this century there was initially an equal interest in environmental and medical aspects but the major discoveries by Pasteur, Koch and many others shifted the focus to pathogenic microorganisms. A summary of the major historical findings (Table One) on the IRB leading to the current level of understanding is summarized in the following table. These dates are based on the publication by Ellis[i] in 1919 that marked the end of an era of studies on the IRB.

Table One
Historical Development of Understandings on the Iron Bacteria

Year	Author	Topic
1838	Ehrenberg	Observations of infusates containing microorganisms
1843	Kutzing	General account of iron-rich living slimes
1878	Rabenhorst	Descriptive nature of slimes
1879	Zoff	Description of *Crenothrix*
1888	Winogradsky	Description of iron bacteria
1892	Sauvage	Description of *Cladothrix*
1893	Kendal	Iron ores in Great Britain
1895	Fischer	General account of bacteria in waters
1895	Rössler	Culture of *Crenothrix*
1897	Migula	Description of *Gallionella*
1903	Adler	Therapeutic value of iron bacteria
1904	Brown	Encrustations in pipe lines
1910	Molisch	First account of the iron bacteria
1911	Herdsman	Organic origin of iron and magnetic ores
1911 - 1915	Ellis	Comprehensive of the various iron bacteria

Ellis in the book "Iron Bacteria" published in 1919 made a number of statements that are pertinent to the development of the IRB-BART tester. These are listed below:

> "The capacity for abstracting iron from waters in which they live and collecting it in the form of ferric hydroxide on their surfaces is possessed by various representatives of most classes of microorganisms that inhabit fresh waters"

> "From the practical standpoint, a study of the habits and peculiarities of the iron bacteria is one which cannot be ignored by those engineers and chemists whose work lies in the supervision of water reservoirs"
> "the second end in view is to reach the notice of the water engineer and the analytical chemist, and perhaps to elicit a measure of sympathy for the endeavors of the biologist"

Ellis's work went unrecognized as having a high priority and much of the development of technologies in microbiology in the twentieth century focused on species of recognized cultivable pathogens. As a result the development of microbiology became focused on techniques to recover and identify pathogenic microorganisms at the species level and beyond using refined molecular and genetic tools. Concurrently, environmental microbiology entered a state of decline until the very end of the twentieth century when sustainability and environmental-risk impacts became a significant concern. The rapid growth of molecular and genetic tools in the last two decades has meant that the specificity for identifying very specific strains has a high level of precision. These developments caused a distortion in the overall approaches to the determination of the causal agents for environmental impact events. The outcome of this was that little attention was paid to the development of broad spectrum determination of IRB activities causing specific nuisance events.

The obsession with micro-cellular studies has denied to some extent the recognition of the importance of macro-community structures in microbiology. This coupled to the lack of interest in the dynamics of the biosphere at the microbial level has led to little progress having been made. For example, the 16th edition of the Standard Methods for the Examination of Water and Wastewater[ii] which included 37 references of which 11 related to the IRB. Most of these references relate to the work of Starkey, Wolfe and Mulder but there was not a significant attempt to improve the examination methods for the IRB. Requests to become involved in the rewriting of section 918 were rejected since the AWWA/APHA required all formulations and methodologies regarding the BART technology to be released. That would have compromised the patent and the section on iron and sulfur bacteria remains effectively unchanged to the 20th edition (2000). In 1995, the AWWA released a revised manual M7 dealing with problem organisms in water. Chapter two[iii] was devoted to iron bacteria as section 9240 but here examination was still restricted to direct and diluted microscopic methods (appendix E). Semi-quantitative techniques are discussed in appendix E under item B.1 using Fe amended HPC medium but are considered to be of little value. The IRB-BART is referred to in B.2 but is not specifically identified.

For the IRB-BART tester, a search began in 1971 for a suitable test method that could be applied both in the field and laboratory that would detect iron bacteria. It was recognized that the iron bacteria played a major role in the plugging of water wells and pipelines but there was little understanding of the relative significance of the bacteria compared to the known and documented chemistry also involved in plugging. In 1978 a review was undertaken reviewing the identification, cultivation and control of iron bacteria in ground water[iv]. This

paper became one of the commonly cited papers upon which much of the current researches on iron bacterial biofouling in water systems are based. By that time there was recognition that the iron bacteria were, in fact, a broad spectral group of organisms that could not easily be cultured using existing laboratory techniques using a defined procedure. Attention was now being paid to nuisance bacteria infesting many natural and engineered systems and by 1986 this recognition was recognized by the American Water Resources Association when they held an International Symposium on Biofouled Aquifers: Prevention and Restoration sponsored by the Environmental Protection Agency. This symposium brought together a range of disciplines including microbiology, engineering and geology and provided a fertile exchange of ideas. An understanding was generated of the complex inter-relationships that exist in biofouled situations including colonization mechanisms[v], formation of biofilms[vi] and the challenge of monitoring methods[vii]. It became clear at the symposium as well as at a precedent think tank[viii] that the evaluation of microbes in ground water is made that more challenging because of the following factors:

- Much of microbial activity occurs within the biofilms attached to surfaces and not in the water which means that the water sample may not be representative of the activity associated with the biofilms growing attached to the surfaces if they are not sheering in any manner
- Microbial activity occurs most commonly as communities of different species that function interdependently
- Much of the microbial activity in the water is of a biocolloidal nature[ix] in which the microbes are contained within a polymeric matrix of bound water. This area of understanding is now beginning to change in very fundamental ways the manner in which water can be viewed
- Relatively few microorganisms in the aquatic ecosystem are planktonic and freely suspended with unbound (free) liquid water
- Because of the above factors, a water sample may not reflect in it's composition the microbes retained within a biofilms attached to surfaces particularly if none are released into the sample
- Examination may be compromised by the biocolloids restricting microbial activities within the test procedure.

A culmination of the 1986 AWRC symposium was a broad realization that suitable field test for iron bacteria did not exist and the suitability of laboratory tests was severely restricted to direct microscopic examination and various agar spreadplate techniques both of which had a limited ability to detect the full range of iron bacteria in water.

A major review of the microbial iron cycle was published in 1983 dealing with the basic science aspects involved in the cycle[x] Rising from these conclusions was the need to develop a suitable field test instrument for detecting iron bacteria. This was achieved with the development of a patent[xi]. The development phase before the completion of the patent process began with the development of a functional testing system for iron related bacteria (IRB). "Iron Related Bacteria" was selected as the term since studies revealed that the traditional approach of separating the iron oxidizing[xii] from the iron reducing

bacteria as two distinct groups of bacteria[xiii] [xiv] may be erroneous in the cases where the same species or consortia of bacteria are capable of both oxidative and reductive functions with respect to iron. It was therefore considered that any iron bacteria presence test proposed that would have to detect both of these activities[xv] but in practice it was found that the earlier tests lacked precision. The concept of the BART rose directly from a number of basic concepts critical to the growth of microbes. Through floating a low-density ball at the fill line of the test tube containing the water sample it was found precision was improved and a routine identification of different reactions could be obtained.

Prototype BART testing for the IRB was begun in 1987 initially using 10ml of water sample. Poor precision continued until the water sample volume used for testing was increased to 15ml whereupon the BART test generated stable patterns of reactions and activities with precision. The term "reaction" was taken to relate to the manner in which the indigenous microbes within the sample changed the observable colors (from the control negative sample) and developed growth forms (such as slime rings, biocolloids, threads, dense gels and floating plates) in the liquid medium. The term "activity" was taken to relate to the time lag before the reactions became observable in the test instrument (BART tester). The hypothesis was generated based on the concept that the shorter the time lag to the observation of a reaction then the more active the microbial population in the sample under the conditions of the test. The first reports on the use of the IRB-BART were in 1990[xvi] [xvii] [xviii] [xix]. At that time the BART was referred to as biological activity test (BAT) but this name could not be used as the trademark due to conflicts with other users while this problem did not exist with the name BART. Between 1988 and 1992, the concepts originally applied to the IRB-BART were adapted to increase the range of potential BART testers. The time frame (Table Two), target bacterial community, name of the BART and the final status are listed below:

Table Two
Development of the BART testers since the development of the patent

Time frame	Bacterial community	BART name	Status
1986 - 1990	Iron Related Bacteria	IRB-BART	Retailed from 1989
1987 - 1990	Sulfate Reducing Bacteria	SRB-BART	Retailed from 1990
1988 - 1992	Slime Forming Bacteria	SLYM-BART	Retailed from 1990
1991 - 1993	Pool Fouling Bacteria	POOL-BART	Commercial Failure
1988 - 1993	Sheathed Iron Bacteria	SIB-BART	Withdrawn 1993
1989 - 1993	Urinary Tract Infection	UTI-BART	Withdrawn 1993
1990 - 1995	Fluorescent Pseudomonads	FLOR-BART	Limited Sales
1990 - 1995	Heterotrophic Aerobic Bacteria	HAB-BART	Retailed from 1993
1992 - 1994	Cyanobacteria	ALGE-BART	Retailed from 1994
1994 - 1996	Denitrifying Bacteria	DN-BART	Retailed from 1996
1994 - 1997	Nitrifying Bacteria	N-BART	Retailed from 1996
1996 - 1998	Bovine Udder Infections	MILK-BART	On Hold
1998 - 2001	Biochemical Oxygen Demand	BOD-BART	In Progress

2.1 Functional Approach Selection

The basic premise for the IRB-BART tester now being presented for verification is listed in section 3 below. The functionality of the BART was described in detail (pages 273 – 315) in the book "Practical Manual of Groundwater Microbiology" published by Lewis Publishers in 1993[xx]. A further expansion of the use of the IRB-BART was described by Smith[xxi] in 1995 and Mansuy[xxii] in 1999. An expansion of the concepts was published in 2000 in the book "Microbiology of Well Biofouling" incorporating more of the quality management data applied to the BART testers. This can be found on pages 137 - 280[xxiii]. Aspects of the relationship of the reaction patterns for the IRB-BART tester to the bacterial community identification are discussed in the book "Practical Atlas for Bacterial Identification". Here there is a discussion of community structures (consortia, pages 131 – 138), the different reaction patterns that can be commonly observed (pages 177 – 186) and a summary of the BART reaction patterns and the interpretation of the time lags (pages 187 – 195)[xxiv]. The concepts for which claims can be made that are also universal to all of the BART testers besides are the IRB-BART tester are listed in the ensuing section 2.2:

2.2.1 Volume of water sample to be used.

From the experimental studies it was found by experience that the most suitable volume of water sample was 15ml.in the test vial commonly employed for the BART tester directly. The vial has an overall height of 89.5mm (uncapped), a base inside diameter of 9.8mm and inside top diameter of 20.5mm with a maximum volume of 30.5ml with a fill line etched on the outer surface of the vial at a height of 56.3mm above the base. The thickness of the vial when constructed out of a medical grade of high clarity polystyrene is 1.9mm. The fill line marks the meniscus for 15ml of water sample when added with the presence of a floating intercedent device (floating ball) that is spherical (see claim 2.2.2) and floating 80% submerged in water having a density of 1.0. The ball has a volume of 2.96ml and when combined with the 15ml water sample leaves a headspace volume of air of 12.58ml including 2.52ml of oxygen. When capped with a polypropylene cap, the text chamber is essentially sealed from the outside environment and for the inner test vial in which the examination of the sample is conducted. In a laboratory, the test vial can be placed in a suitable standard test tube rack for incubation and observation. In this format, the tester is referred to as the "Lab IRB-BART". The outer base diameter of the test vial is 24.0mm which makes the device somewhat unstable if not supported since the height to the cap is 89.5mm creating a high center of gravity particularly when charged with a water sample. In the field use of the IRB-BART tester, the inner test vial is contained within an outer test vial to provide additional security. Additional security includes:

- Double walled protection of the user from odors generated by the test
- Containment of any leakages of liquids from the inner test vial during the test period (incubation) and improved security during final disposal
- Greater protection of the inner test vial during transportation to the site where the testing will be performed

- Convenient labeling of the outer test vial with information concerning the test while the inner test vial simply has the red color encoded cap showing that it is an IRB-BART.

Additionally, the outer test vial can be used as a convenient sample collection container. It has a 31.5mm inner diameter at the base that rises to 33.5mm as the top inner diameter with an overall height of 95.3mm and a volume of 75ml which would be enough sample volume to undertake 5 BART tests.

It is claimed that the use of 15ml gives an adequate volume of water to entrap a sufficient a range of bacteria occurring in biocolloids, sloughed suspended biofilm materials and in the planktonic form to ensure that the targeted IRB communities will thrive within the IRB-BART tester. Technician error in filling the vial with water sample is reduced by recommending that the water sample is pipetted into the vial using a 10ml pipette. This restricts the filling error to ±0.2ml. While pipetting in the laboratory setting would achieve this level of accuracy, in the field under cruder conditions a manual filling of the vial may commonly occur. For the manual fill, the normal variation in the meniscus of the water sample to the fill line would be 2mm. This variation would translate into a ± 0.7ml variance (5%) in the amount of water sample. Consequently the claim would recommend that a 10ml pipette be used for laboratory examinations using the lab IRB-BART testers. For the field application of the IRB-BART testers then a cautionary note would be included that where the IRB-BART test vial is filled manually then care should be taken to ensure that the final water sample level is within 2mm of the etched fill line on the BART tester.

Claims relating to the filling of the IRB-BART testers with water sample being proposed for verification in this document are:

- That 15ml of water sample is added to the IRB-BART tester to initiate the start of the test.
- In the laboratory setting, it is recommended that a sterile 10ml pipette would be used to dispense the sample as two equal aliquots of 7.5ml. Dispensing of the sample would following accepted aseptic procedures commonly employed by those familiar with the art. The water sample should be dispensed from the fill line position keeping the pipette tip from 3 to 5mm above the ball as the ball floats up. The precision of this dispensing is expected to be ±0.2ml.
- In the field setting where it is not possible to use a pipette to dispense then a manual filling of the IRB-BART tester is permitted provided that this is performed in a dust free clean environment and the level of filled water sample in the BART tester is within 2mm of the fill line mark on the side of the IRB-BART tester.
- The maximum tolerance for error for filling the IRB-BART tester is 5% and the amount of water sample to be tested has to fall within the range of 14.25 and 15.75ml. It is considered that this level of error would not compromise the ability of the IRB-BART tester to detect the targeted bacterial group within the water sample since the aspect ratio (see 2.2.3)

and the diffusion rate of the selective culture medium (see 2.2.5) would not be significantly compromised by this degree of variation.

2.2.2 Use of the Floating Intercedent Device (Floating Ball)

A key component in the claims is the floating ball that floats on the surface of the water sample and restricts the entry of oxygen from the head space into the water sample. This restriction is created by float ball having a diameter of 19.75 ±0.05mm and floating on the water sample at the filled line with a inside diameter of 22.00 ±0.07mm. The movement of oxygen by diffusion around the ball is therefore restricted at the sunken equator of the ball to 73.8mm^2 of total lateral area at the fill line of 380.2mm^2; this reduces the area for oxygen diffusion at the throat between the ball equator and the wall of the IRB-BART test vial by 80.6% to 19.4%. The floating ball has the density adjusted to sink by 80% of its vertical profile into water having a density of 1.0. This would mean that the ball would float with 17.6mm submerged and 4.4mm of the vertical profile out of the water. The upper curved surfaces of the ball that are submerged have a high exposure to the diffusing oxygen from the head space. It is at this site that there tends to be a concentration of aerobic microbial activity and forms of aerobic growth (such as slime rings and biofilm generation) can become concentrated. The color of the float ball is a pure white and these growths can be clearly observed against the surface of the ball. The underside of the ball sits immersed in the water sample under conditions of increasing oxygen stress when there is a significant level of microbial activity from the indigenous microbes under the more reductive conditions created here. The lower curved surfaces of the float ball can also form sites for the attachment and growth of some microorganisms causing slime formation and/or discoloration of the ball. At the same time these surfaces can also cause elevating gas bubbles formed by fermentation to become temporarily attached to the surfaces where commonly the gas bubbles will, if not degraded, rise to form a foam ring around the ball that becomes easily recognized.

Claims relating specifically to the float ball of the IRB-BART testers with water sample being proposed for verification in this document are:

- The float ball generates on the upper hemispheric surfaces that are coated with a water film that forms a site for aerobic bacterial growth that can become observable.
- The float ball generates on the surfaces of the lower submerged hemisphere conditions where gases produced by fermentation deeper down in the inner test vial can collect. Gases can then continue to move upwards to form foam around the ball. Additionally some microbes can, under these more reductive conditions, cause low density observable growths and products of growths to collect on theses surfaces.
- By floating the ball 80% submerged in the water sample, there is a restriction in oxygen entry into the body of the water sample under test and this can encourage the growth of microbes that function under reductive (anaerobic) conditions deeper down in the test vial.

- In floating the ball on the water sample being tested using the IRB-BART, reductive conditions can arise due to the restriction by 80.6% in the diffusive movement of oxygen down into the water sample. This, when there is significant microbial activity, results in a stratification within the water sample being tested with reductive conditions at the base, oxidative conditions above the equator of the ball and a redox front at the interface between these events.
- The ball, as a result of the claims given above, creates within a single test a series of lateral environments having different parameters and changing ORP values from reductive at the base to oxidative at the surface. This test through the admission of the float ball generates in a single test that has a greater variety of environments than are usually presented in microbiological test procedures.

2.2.3 Generation of an Aspect Ratio

One major claim relating to the patented IRB-BART system is the creation by the floating ball of an intercedent device that in restricting oxygen entry hastens the formation of a reduction-oxidation gradient when there is any significant microbial respiration within the water sample being tested using the IRB-BART. The aspect ratio, as applied to the BART tester, relates to the surface area through which the oxygen can diffuse from the headspace into the water, and the volume of water that receives the oxygen. For the IRB-BART tester without a float ball then the aspect ratio would be 1: 3.95 for the exposed water surface area: volume. This would mean that for every square centimeter of surface the oxygen could diffuse through there would be almost four ml (cm^3) of the water sample volume underneath. Under these conditions there would be a considerable ability for the oxygen to dissolve into the surface water film and diffuse down the water column. In the IRB-BART tester the constriction of oxygen diffusion is created at the equatorial point when the ball is at its widest and this shrinks the surface area down by 80.6% to 73.8mm^2. This would thus exaggerate the aspect ratio beneath the equatorial region of the float ball. The minimum acceptable volume of the water sample is composed of 1.24ml above the base of the ball, 12.85ml in the water column beneath the equator of the ball and 0.76ml in the basal cone of the IRB-BART tester for a total volume of 14.85ml including the selective culture medium crystallized on the floor of the basal cone. The aspect ratio for the IRB-BART test calculated from the lateral equator of the ball is therefore increased from 1: 3.95 (with no ball) to 1: 20.1 (surface area reduced by the constriction at equator to 73.8mm^2 and the volume under the equator calculated to be 14.85ml including allowance for the crystallized selective medium). The aspect ratio using the ball therefore causes a fivefold increase which further restricts oxygen entry and allows the oxidation reduction potential gradient to materialize, stabilize and shift upwards in the event that microbial respiration occurs in the sample during the testing period (incubation). During the development of the IRB-BART between

1986 and 1991 the early float ball was a hollow polypropylene ball with an outside diameter of 18mm. This would mean that when the area was calculated for the water surface area (between the wall of the BART and the equatorial region of the ball) was 125.7mm^2 which would have been 70.3% larger than the new IRB-BART testers introduced in 1992 with the new larger white foam ball. This larger surface area at the equator would have impacted on the aspect ratio that was originally 1: 11.8 by increasing that to 1: 20.1. It was found during that period of development that the IRB-BART reactions did not display stable lateral activities in the water sample column that could clearly be associated with the formation of an ORP gradient. Experimental modifications using floating plastic disks revealed that an aspect ratio of at least 1: 15 was needed to stabilize these events for a water column being tested in the established IRB-BART test vial.

Claims relating to the aspect ratio of the IRB-BART testers containing a 15ml water sample that are being proposed for verification in this document are:

- That an aspect ratio of equatorial surface area between the float ball and the wall of the IRB-BART tube has been set at 1: 20 so that, where there is indigenous microbial respiratory activity in the sample, an oxidation-reduction gradient forms along the vertical axis of water column. This gradient would be highly oxidative above the equator of the ball and move progressively more reductive down the column to become very reductive at the base of the IRB-BART test vial.
- That the generation of an oxidation – reduction gradient within a water sample incubated in the BART as a result of indigenous microbial activities would be created by a series of lateral environments supportive to different groups of IRB.

2.2.4 Capping the IRB-BART tester

The IRB-BART tester vial is capped with a red single turn screw polypropylene cap. Once the water sample has been added to start the test, the cap is screwed down firmly to restrict the admission of air to the test vial and also reduce the risk of off-odors arising from the microbial activities escaping into the atmosphere. Once screwed down the cap provides a water-tight seal in the event that the IRB-BART vial charged with water should be accidentally knocked over.

2.2.5 Rates of Diffusion of the Selective Culture Medium

One major feature in the use of the IRB-BART tester is the ability to select the type of bacterial community that could be detected. This is achieved using the WR[xxv] selective culture medium based on the original agar formulation (excluding the agar) that is dried onto the conical base of the IRB-BART test vial. Once dried, the modified WR medium is intensely hydrophilic and can under highly humid conditions take up water relatively quickly. To prevent this, all IRB-BART testers, once they have passed the quality management procedures, are packaged in aluminum foil

sealed pouches that have a very low permeability to water. The medium selected for use in the IRB-BART relates to the iron related bacterial communities.

The general effects of adding the water sample into the IRB-BART tester on the selective culture medium in the base is detailed below. It should be noted that once the water sample has been added and the cap screwed firmly down then the IRB-BART tester should not be shaken or inverted. It should also be noted that there will be minor variation in these events depending upon the interaction between the chemistry of the water and the nature of the microbes in the water sample:

- Event one, a diffusion of the medium occurs from a colored cloud in the conical base of the IRB-BART that may extend as much as 1 to 3mm up the vertical side wall of the IRB-BART tester inner vial.
- Event two, a diffusion fronts can be seen rising up the water sample in the IRB-BART test vial. This front usually becomes more transparent and less colored in the upper reaches of the diffusive front.
- Event three, the diffusion fronts dissipates to form an evenly colored solution that would normally be totally transparent where there is no microbial activity.

The indigenous microbial population, when activated by the WR diffusion front, may interfere with the manner in which these events occur. Commonly with the IRB-BART the first divergence is the formation of either: cloud-like growths, an occurrence of increasing general turbidity, gassing or significant color changes in the water sample in the IRB-BART in a manner not typical for a straight diffusion of the WR medium.

Claims relating to the selective culture medium used in the IRB-BART testers with water sample being proposed for verification in this document are:

- That the specificity of a given IRB-BART for particular bacterial communities is primarily controlled by the election of the modified WR selective culture medium crystallized into the conical floor of the IRB-BART tester vial.
- The reactions and activities observed in a BART tester may be interpreted in a semi-quantitative and semi-qualitative manner to provide information on the size and form of the IRB community so detected.

2.2.6 Generation of a Oxidation – Reduction Potential (ORP) Gradient

From sections 2.2.2 and 2.2.3 it has been claimed that the IRB-BART tester set up in a manner that follows the protocol described has the potential to develop an ORP gradient due to the aspect ratio created by the floating ball within the IRB-BART tester. In a condition where there is no detectable IRB activity then the water sample may not, when placed in the IRB-BART tester, begin to generate an oxygen demand due to the lack of any respiratory activities since there are no indigenous microbes able to become active in the water sample. An ORP gradient is most likely to be

generated when there is a significant biological respiration that begins to remove the dissolved oxygen from the water sample under test. Once the removal of oxygen by respiration and other biological activity exceeds the ability for oxygen to diffuse down into the water from the "throat" created at the equator of the ball then the oxygen concentration will decline to establish a series of laterally stratified ORP zones with the more oxidative regions further up the vertical profile of the water sample. It is well established that different microorganism will function most efficiently at different ORP values[xxvi].

Claims relating to the generation of an ORP gradient in the IRB-BART tester that have become biologically active (when the water sample is added and incubated) being proposed for verification in this document is:

- That an oxidation – reduction gradient will form in the IRB-BART tester where there is biological activity that reduces the oxygen concentration in the water column.
- Specific IRB communities targeted by the IRB-BART test are likely to locate at particular and characteristic places along the vertical profile in the IRB-BART test in response to the establishment of the ORP gradients.
- That a unique feature in the IRB-BART tester is the ability to generate a range of environments within the oxidation and reduction gradient in biologically active water sample that allows the IRB to be recognized by activity at a more specific site than just in the total length of the water column of the IRB-BART tester.

2.2.7 Incubation Conditions for the Testing Period

There is a considerable concern about the incubation temperatures at which IRB-BART test should be kept in order obtain a satisfactory set of data that has a relationship to the potential for the same targeted bacterial community to be active in the natural environment[xxvii]. In natural waters a normal temperature range that can be expected to support some level of bacterial activity varies with the geological setting. For the IRB-BART tester it is normally recommended that tests be performed at controlled room temperature that can normally be expected in the mean of the operating temperature would be22^{o}C. This temperature is adequate to allow the activity of psychrotrophs and mesophiles but not the psychrophiles that normally cannot be active at above 18^{o}C. There is clearly a trade off in selecting a wide temperature range for the IRB-BART test that is undertaken to meet the needs of convenience. Such a variation becomes of less significance where there are comparative tests being undertaken between water samples taken at different times under various conditions. If the water samples were taken from sites having a temperature of below 8^{o}C then consideration should be given to running duplicate IRB-BART tests at 8±1^{o}C as well as the set at room temperature. The former temperature (8^{o}C) would tend to encourage the growth of the psychrophilic bacteria while the higher temperature would indicate the activity of the psychrotrophs and the mesophiles. For hot waters having

temperatures of between 50 and 65°C then the incubation temperature can be set at 54±1°C. It should be noted that the maximum storage temperature for the IRB-BART tester under exceptional conditions should not exceed 58°C.

Claims relating to the incubation temperature used for the IRB-BART testers being proposed for verification in this document are:

- For testing using the IRB-BART testers, it is recommended that testing be conducted at normal room temperature (ranging from 19 to 26°C) with a mean in the range of 21 to 23°C.
- For laboratory testing using the IRB-BART testers, it is recommended that a duplicate set of IRB-BART testers be incubated at 8°C when the original ambient temperature of the water sample when collected was at less than 8°C.
- For laboratory testing using the IRB-BART testers, it is recommended that a duplicate set of IRB-BART testers be incubated at 54°C when the original ambient temperature of the water sample when collected was over the range from 50 to 65°C
- No incubation studies showed are conducted at temperatures exceeding 58°C since the integrity of the IRB-BART tester may be compromised.

2.2.8 Recognition of a Reaction as a Positive Detection

There are two stages in the gathering of critical data from incubating IRB-BART testers. During the incubation, commonly at daily intervals, the IRB-BART testers are inspected visually for any activity that could be associated with IRB activity. Inspection may involve lifting the IRB-BART tester up so that a diffuse light can pass through the tester to show any form of reaction associated with growth, color change, development of turbidity or the production of gas. For the IRB-BART tester there is a list of coded reactions that may be considered to be a positive indication of activity for the IRB. These are discussed in detail in section 2.4 and section 3.1.3. Over the incubation temperature range of 19 to 25°C it is common for all of the reactions to be observed in the IRB-BART tester will be observed by the tenth day. All reactions observed that are relevant to the detection of a positive reaction in the IRB-BART tester need to be recorded with the date on which that reaction occurred. This information is then used in the determination of the time lag (section 2.2.9) and the reaction pattern signature (section 2.2.11). With the IRB-BART testers there may be a sequence of reactions that will be observed following the first reaction and these should also be written down in code form on the standard field or laboratory IRB-BART tester data entry sheet. Normally all secondary reactions will be observed within four days of the first reaction being recognized.

2.2.9 Determination of the Time Lag to a Positive Detection of a Reaction

While the reaction is determined by the recognition of an activity in the IRB-BART tester as being typical of one of the target IRB groups being determined, the time at which this occurs since the start of the test gives an indication of the level of activity. All of the information relating to the IRB-BART tester being used on a specific water sample needs to be recorded. The methods by which this should be performed are described in section 2.2.10. At the time that the IRB-BART tester is first charged should be recorded by calendar date and hour using the twenty four hour clock. When the first positive reaction is observed it should be recorded on the standard field or laboratory IRB-BART tester data entry sheet by entering the time (to the nearest hour) and the code allocated to the reaction observed. The time lag is the difference between the time that the IRB-BART tester was first set up and the time at which the first positive reaction was observed. This difference is given in days to one decimal place. For example, the first reaction was observed at 16:30 on the day following the start up of the IRB-BART tester which was began at 08:30 then the time difference would be 1day and 10 hours which would be shown as 1.4days. The targeted bacterial groups are considered to be absent from the sample when no reaction has been observed by day ten.

2.2.10 Semi-Quantitative Evaluation Mechanisms, Aggressivity

The aggressivity of the targeted IRB in the water sample is used to provide a guide to evaluating the activity level of the bacteria rather than the number of cells (population commonly presented as colony forming units per ml). Viable counting of bacterial populations has in the last two decades generated serious concerns with respect to the use of agar spreadplate techniques [xxviii] generating too narrow a spectrum of colonial growth from the targeted bacterial community. The use of aggressivity is herein claimed to be valid since it reflects the ability of the intrinsic indigenous bacteria in the water sample to grow in the IRB-BART tester under the variety of environmental conditions created by the formation of both an ORP and a selective culture medium gradient within the water column in the tester. Aggressivity is therefore a measure of the activity of the targeted bacterial group rather than the calculated number of cells. Aggressivity therefore more closely parallels the assessment for the total ATP (adenosine triphosphate, a high-energy phosphorus compound) that is commonly found in metabolically active systems. This is because both relate to biological activity and not cell numbers/populations.
In the development of the IRB-BART tester, experiential evidence was the primary driver in establishing the categorization of the time lag into levels of aggressivity. These categories are defined in the Table Three.

Table Three
Definition of Aggressivity and its Relationship to Time Lag
For the IRB-BART tester

Aggressivity	Definition	Relationship to Time Lag
High >4.1 days	There is an observed reaction that occurs quickly after the start of the IRB-BART tester being incubated indicating that there is either a very large or very active population of the targeted bacteria	Shorter than the first critical time lag marker event
Medium 4.1 to ≤8.0 days	There is a significant delay before the recognition of the first reaction after the start of the IRB-BART tester being incubated indicating that there is either a moderately active or modest population of the targeted bacteria	The time lag falls between the first and second critical marker event
Low >8.0 days	There is a prolonged delay before the recognition of the first reaction after the start of the IRB-BART tester being incubated indicating that there is either a very small population of the targeted bacteria or that they have a low level of activity and are not able to become very active in the IRB-BART tester.	The time lag falls between the second and third critical marker event
Not Detected >10 days	There was no observable reaction indicating that none of the bacteria from the targeted group in the water sample were able to be active in the IRB-BART test due to too small a population threshold or too metabolically impaired to become active	Time exceeds the third critical marker event and no reaction has been observed in the BART tester.

Notes: The first critical marker event is that time interval from the start of the incubation of the IRB-BART tester when it is considered that there can no longer be a highly aggressive target bacterial community in the water sample being tested. The second critical marker event is that time interval from the start of the incubation of the IRB-BART tester when it is considered that there can no longer be a moderately aggressive target bacterial community in the water sample being tested. The third critical marker event is that time interval from the start of the incubation of the IRB-BART tester when it is considered that there can no longer be a target bacterial community of significance in the water sample being tested. The above interpretation is based upon incubation at room temperatures

Claims relating to the determination of the aggressivity of the targeted bacteria in the water sample when tested for using the IRB-BART tester being proposed for verification in this document are:

- That the IRB-BART tester has the ability to define the levels of aggressivity of the target bacteria in a water sample into three categories (high, medium and low) on the basis of their level of activity in an incubated BART tester charged with the water sample recorded as the time lag given in days to one decimal place.

2.2.11 Semi-Qualitative Evaluation Mechanisms, Reaction Pattern Signature

While the IRB-BART testers are capable of assessment of the activity level for the targeted bacterial communities within a scale of aggressivity involving four levels (high, medium, low and absent), it is also possible to achieve a semi-qualitative determination of the recognized bacterial communities by the sequence of reactions that are recognized during the testing period. It should be recognized that different bacterial communities may function effectively and interdependently within different focal sites within the tester. Using the standard field or laboratory IRB-BART tester data entry sheet, the chronological sequence in which the reactions can be obtained by applying the coded reactions listed on the sheet. This string of codes can be converted into a single reaction pattern signature (RPS) that may then be used to identify the nature and composition of the detected bacterial community in the water sample. The following rules apply to establishment of the RPS:

- The reaction codes are listed in strict chronological order from left (earliest) to right (latest).
- Reaction codes observed on different days are separated by a single dash (-) to show by the number of code clusters that occurred as discrete reactions on each day.
- Where more than a single reaction occurred on the same day of incubation a comma (,) is inserted between the concurrent codes. This indicates that both reactions occurred essentially at the same time.

As an example of the use of the RPS, the following code FO, CL – GC – BR,BL in an IRB-BART would mean reference to the BART data interpretation chart (see section 2.2.12) that the targeted bacterial community in this water sample included iron related bacteria that caused a foam (FO) and clouded growth reaction (CL) on the same incubation date followed on a later day by a green cloudy reaction (GC) which was followed by brown ring (BR) and a blackened liquid (BL) after at least one more day. Table Eight (page 32) addresses the differentiation of the IRB into five major family groups on the basis of the sequence of the reactions.

Claims relating to the determination of the RPS of the targeted bacterial community in the water sample when tested for using the IRB-BART tester being proposed for verification in this document are:

- That the semi-qualitative nature of the IRB community detected by the IRB-BART tester during the incubation of a charged water sample can be

inferred by the reaction pattern signature generated during the routine monitoring of the BART tester during the incubation period.

- The RPS so gathered using the IRB-BART tester allows information to be interpreted relating to the nature of the various bacterial species forming parts of the IRB community detected in the water sample during the IRB-BART tester incubation of the sample.
- That the interpretation of the RPS can be gainfully used in the management, diagnosis and treatment of nuisance IRB events in water.

2.2.12 Record Keeping and Preliminary Interpretation

There are three levels of record keeping for the IRB-BART tester being proposed for verification. These include:

- Field BART tester data entry sheet
  The field BART tester data sheet is set up with fourteen columns representing in column one the type of BART tester being used while the ten rows to the left represent each of the ten days during which activity can be observed for each BART tester. The last two columns are devoted to a calculation of the time lag (in days only) and then a summarized reaction pattern signature (see section 2.2.11 for specific details of the format). The final column gives the aggressivity using the four scaled approach described in section 2.2.11. A single row is devoted to each particular BART test being conducted on the specified water sample. Only data relevant to that water sample may be entered onto that specific sheet. Entries show the reaction codes that are recognized as new in the column set aside for that date of the testing. There is space in each box for up to three reaction codes to be handled on any given day. The top of the field BART data entry sheet includes boxes for giving details on the location, sampling method, and origin of the water sample used to conduct the BART tests recorded on the sheet.
- Laboratory BART tester data entry sheet
  The laboratory BART tester data sheet is set up with fifteen columns representing in column one the type of BART tester being used while the ten rows to the left represent each of the ten days during which activity can be observed for each BART tester. The next two columns are devoted to a calculation of the time lag (in days only) and then a summarized reaction pattern signature (see section 2.2.11 for specific details of the format). The time at which the positive detection of the first reaction occurred is also included given the hours and minutes using the twenty-four clock. In the penultimate right hand column is devoted to the projection of the log population in cfu/ml based on the BART data interpretation chart described below. The final column gives the aggressivity using the four scaled approach described in section 2.2.11. A single double-wide row is devoted to each particular BART test being conducted on the specified water sample. Only data relevant to that water sample may be entered onto that specific sheet. Entries show the reaction codes that are recognized as new in the column set aside for that date of

the testing. There is space in each box for up to three reaction codes to be handled on any given day. The top of the field BART data entry sheet includes boxes for giving details on the location, sampling method, and origin of the water sample used to conduct the BART tests recorded on the sheet.

- BART data interpretation chart (reference use only)
  The BART data interpretation chart can be employed by technicians and users in order to obtain standard information relating to the interpretation of the BART testers at room temperature. This chart is in two parts with the upper part used to interpret the time lag data into aggressivity and possible log population cfu/ml. The lower part of the chart gives a list of all of the accepted reaction codes for the various BART testers in production. The center rows of the chart provide a conversion from log to arithmetic population. Only recognized reaction codes may be entered into the data sheets as a part of generating the RPS. This chart is used for reference purposes only and should not be used to record and compile data from individual BART testers.

Claims relating to the recording and interpretation of the activities of the targeted bacterial community in the water sample when tested for using the IRB- BART tester being proposed for verification in this document are:

- All information relating to the application of the IRB-BART testers in the field should be recorded on the standard Field BART tester data entry sheet
- All information relating to the application of the IRB-BART testers in the laboratory should be recorded on the standard Laboratory BART tester data entry sheet
- Any interpretation of the RPS and time lag data should performed using the standard BART data interpretation reference chart
- All of the BART data to be interpreted in this manner should have been incubated over the normal range of room temperatures under conditions as described for the standard operating procedures for the conductance of BART testing

2.2.13 Security in the Application of Field BART tester

From the beginning of the development of the IRB-BART tester in 1987 there were a number of problems that emerged in the remote application of the BART testers in the field. These may be summarized as relating to:

- The outer diameter at the base of the BART test vial was 24mm and the height when capped 92mm. Because of this excessive height to width, an unstable condition was created whereby the test vial could be knocked in any manner and it would follow over particularly since the center of gravity of the filled BART would be approximately 34mm above the base. It was not uncommon for the vials to fall over and create a domino effect as they struck neighboring BART testers.
- The nature of the test vials used at the start of the development of the test protocols was such that there were fractures in the side walls and

incomplete fusion of the conical base to the vial. The net effect of this was that there was slow leakages (e.g., 0.01 to 1.0ml per day) and catastrophic failures (e.g., 15ml in one day). At one stage these failures reached 1.5% of the IRB-BART testers charged with water samples. This created a severe hygiene risk (due to cultured microbes escaping from the compromised IRB-BART testers).

- One almost inevitable bi-product of the growth of microbes in the IRB-BART tester is the generation of odorous and/or volatile compounds that were able to creep out mainly around the cap: wall seal interface. These odors could become so severe that, on one occasion, a wing of a hotel within which a variety of BART testers were being used in one room by the field crew had to be closed down and the area ventilated.

Clearly all of these events created unacceptable circumstances and a modified field IRB-BART tester was developed to prevent these risks from developing. This was achieved by taking a number of steps to correct the problems. The most significant was to include in the field IRB-BART tester an outer red screw capped vial that was large enough to hold the inner BART tester vial. The dimension for the outer BART tester vial was set with a 34mm diameter base and a 97mm height. The inner BART tester vial was fitted tightly into the outer vial and flanges in the outer cap retained the inner BART tester vial in a central and locked position. The outer BART tester vial provides the following advantages: (1) all odors generated by the BART tester during incubation are retained; (2) any leakages from the inner BART tester vial are contained by the outer vial; (3) the inner BART tester vial is protected from damage by sudden physical stresses; and (4) the outer BART tester vial is much more stable when knocked and much less likely to fall over.

Additional steps were also taken to improve the security of the inner and outer BART tester vials. These were: (1) develop an injection mold that would ensure that the junctions to the conical base of the inner BART tester were thick enough to reduce the risk of fracture and failure; (2) upgrade the quality of the polystyrene to be used to a medical grade with a high clarity to ensure integrity of the plastic tube; (3) elect a higher grade of polypropylene for the caps of both the inner and outer vials to ensure a better fit and seal to the upper lips of the vials when screwed down firmly; and (4) place inner concentric seal flanges on both the inner and outer test vial caps to ensure that the inner BART test vial is pulled up and out of the outer BART tester vial when the outer cap is unscrewed, and that for the inner cap a flange tightly closes around in the inner edge of the inner BART tester vial and seals off the environment inside the inner BART tester vial.

All of these changes were fully in-place in 1996 and there has been acceptance of these modifications to improve the security of the BART tester during incubation in the field.

2.2.14 Security in the Application of the Laboratory IRB-BART tester.

In the analytical laboratory setting, there is a desire to conduct the IRB-BART test as economically as possible. The laboratory IRB-BART testers are

provided in a minimum form for the completion of the test. Here the inner vial of the IRB-BART tester is provided without an outer BART test vial and the tests should be performed in a laboratory by technicians skilled in the basic microbiological techniques. The basic criteria that are required for the use of the laboratory IRB-BART testers are: (1) the IRB-BART test should be undertaken in a laboratory that has the ability to undertake microbiological testing following the standard practices commonly employed by those skilled in the art; (2) the IRB-BART testers should be protected from being disturbed by being placed in test tube racks able to accommodate test tubes having a diameter of between 25 and 28mm with supportive support set at a height no lower than 56mm and no higher than 76mm above the floor of the test tube rack. In the event that the IRB-BART tester is suspended then the hole through which the BART tester is supported should be in the range of 26 to 27.5mm (diameter) and this should be raised 83 to 100mm above the surface upon which test tube rack is sitting. This latter test tube rack format is recommended since the row of IRB-BART testers held in the test tube rack may be raised together and observed at the same time.

Claims relating to the format for the IRB-BART testers being proposed for verification in this document are:

- That the field IRB-BART tester provides a secure method for the undertaking of a microbiological investigation of the activity and form of the targeted bacterial groups. The risks of damage to the tester, of leakages of odorous materials or microbes from the containment in the inner IRB-BART test vial are reduced to negligible proportions.
- That the field IRB-BART tester provides a convenient field testing technique that can be set up at remote locations and provide information at-site.
- That the field IRB-BART testers can form a suitable enrichment technique so that when it is returned to a laboratory, confirmatory microbiological studies can be undertaken to obtain microbial cultures for confirmatory studies.
- That the laboratory IRB-BART tester provides a convenient technique for the determination of the bacterial communities and also be treated as an enrichment technique for confirmatory investigations on the IRB.

2.2.15 Disposal Issues for the IRB-BART

There is naturally a concern that spent IRB-BART testers are disposed of in an effective and safe manner particularly where the testers has detected the presence of bacteria in the water sample. The risks from inappropriate disposal are hygiene risks from the incumbent cultures microbes and aesthetic problems particularly relating to odors emanating from the tester and offensive slimes residing in the tester. Recommended practices are that either the spent BART testers are taken to a facility that has the ability to steam sterilize (autoclave) the materials or the testers can be placed in a sealed plastic bag in groups of no more than eight testers. These bags would then be subjected to pasteurization using a dedicated 800 watt microwave operating on HIGH for 50 seconds. This is sufficient

exposure to bring the contents up into the range of 70 to 90^{o}C for a minimum of 10 seconds.
Some field use of the BART testers involves the unscrewing of the caps and throwing the contents into a pail of bleach solution and left overnight before disposal in the garbage. This technique is not recommended.

2.3 IRB as a Target Nuisance Bacterial Group

"Nuisance" is defined in the Concise Oxford Dictionary as: "a thing, person or thing causing trouble or annoyance or anything harmful or offensive to the community or a member of it". Nuisance bacteria therefore are those bacteria which in their presence, growth or activity cause a nuisance condition that causes a definable problem to be generated within a natural or engineered event. The level of the nuisance activity can be indirect to society through negative impacts to the delivery of a product or service, or direct through the infection by nuisance bacteria of members of society causing clinically definable symptoms to be recognized and treated. For the verification of the BART testers the definition is restricted to the nuisance bacteria that have an indirect impact.
"Iron bacteria" used to be the traditional name for the bacteria that accumulate iron beyond their immediate metabolic requirements. Their has been a general differentiation of these bacteria into iron oxidizing and iron reducing groups depending upon whether the product iron was in the ferric (oxidizing) or ferrous (reducing) form[xxix] respectively. Of more significance was the iron oxidizing group in which significant surplus ferric product was deposited within and/or around the cell. Originally these ferric deposits were thought to be the product of geochemical process but direct microscopic[xxx] and cultural techniques[xxxi] revealed that these deposits were actually created by microbial activities. These ferric depositing bacteria were found to exist in three forms[xxxii]: (1) ribbon formers in which the ferric surplus was deposited in a ribbon-like outgrowth; (2) sheath formers in which the bacterial cells lived within slime tubes onto and in which the ferric compounds accumulated; and (3) slime formers in which the ferric compounds accumulated within biofilms to mature into encrustations, nodules, rusticles[xxxiii] and tubercles. In research conducted in the 1980 on iron bacteria it was found that the same bacterial communities causing the production of ferric compounds oxidatively could also under reductive conditions reduce the ferric iron back to the ferrous form[xxxiv]. Given that the same bacteria community was found to be capable of both oxidizing and reducing iron it was proposed that the term "iron related bacteria" be employed[xxxv]. This has now become a broadly accepted term.
In essence the IRB are complex and reactive community of microbes that create a ferric-rich habitat under oxidative conditions.
In practice the IRB create a range of problems associated with the "throttling" down of water flows through pipes and porous media creating plugging problems that can totally occlude water flow. The nuisance value relates to increasing energy costs as the pressures to pump given volumes go up, radical slippages in water quality and linked system failures due to associated corrosion events.

2.4 Semi-Quantitative Evaluation

Semi-quantitative evaluation is achieved by an assessment of the aggressivity of the indigenous bacteria in the water sample being tested. The definition of the term "aggressivity" and its generation are discussed at the theoretical level in sections 2.2.8 to 2.2.10. The establishment of the link between time lag and the level of aggressivity of the indigenous bacteria in the water sample has been developed through a sequence of events. Major problems in developing these links has been: (1) the inability of the standard agar spreadplate techniques to have an adequate sensitivity to detect as broad a range of targeted bacteria as the IRB-BART testers are able to; (2) the total inability of microscopic, spectrophotometric and laser particle counters to differentiate the forms of target bacteria in the sample; (3) the inability of the analytical techniques such as the adenosine triphosphate (ATP) to differentiate specific IRB groups; and (4) the tendency for target-specific labeled molecular markers to fail to detect cells components unless high sample volumes are employed to avoid unacceptable false negatives. In using the IRB-BART tester for semi-quantitative evaluations there is an intrinsic problem resulting from the greater sensitivity of the IRB-BART tester since it provides a broader range of dynamic environments than other quantification systems. In the generation of aggressivity into a four scaled event for each of the BART testers, there has been developed a broad spectrum approach to the election of the threshold time lags for each event (see section 2.2.10 for definition of the events). The approach that has been adopted in setting these time lag criteria is a combination of the following techniques:

- Sample data comparisons between the time lag and populations recorded using selective cultural practices.
- Determination of time lags achieved for populations of cultured bacteria from the target group. These bacteria may be pure cultures from the American Type Culture Collection (ATCC) or isolates from natural samples known to be dominated by the targeted bacteria
- Extinction dilution series of water samples to determine the impact of dilution on the length of the time lag using the IRB-BART tester
- Experiential evidence garnered by using the IRB-BART testers in the field as a part of the monitoring strategy involving treatment procedures that are likely to impact on the aggressivity of the target bacterial group (e.g., disinfection, rehabilitation)

One major challenge during the early development and marketing of the IRB-BART testers was comparisons with the established techniques. Two examples of this are given below as sections 2.4.1 and 2.4.2.

2.4.1 Dip-Paddle technique comparisons

The agar dip paddles are a simple modification of the agar plate where a thin film of agar is attached to a tray that is then dipped in the water sample to be tested. The agar contains the selective chemical ingredients to optimize the potential for the targeted bacteria to grow as discernable and identifiable colonies that can then be counted and/or identified. In the original development of the IRB-BART tester it was considered by the first distributor (Layne Inc., Kansas City, Ka[xxxvi]) in 1988 – 89 that

the IRB-BART testers would provide a simple, more robust but less sensitive field test to the dip-paddle. When applied in that manner with comparisons with the dip-paddle on the same well water samples it was found by the company that the BART testers recovered bacterial activity in more samples than the dip-paddles did. The dip-paddles were discontinued and Mansuy (1999)[xxxvii] wrote: "we've used BARTs now for many years in the U.S… They are excellent tests… You get better assessment of groundwater microorganisms with a BART than you can with a heterotrophic plate count or microscopic analysis". In 1989 Layne changed the approach to the BART testers considering them to be superior to the dip-paddle and enacted a marketing plan to distribute the BART testers. Negotiations terminated over exclusivity issues. Hach Company (Loveland, Co) then became a distributor the BART testers and accepted the evidence that the BART tester was more sensitive and convenient for the detection of target bacteria in water.

2.4.2 Agar Spreadplate technique comparisons

Hach Company began to distribute the BART testers in 1990 in competition with Layne Inc. The first testers to be distributed were the IRB-, SRB- and the newly developed SLYM- BART products. Initially, Hach did conduct internal comparisons and obtained a similar result to Layne for the dip-paddle comparisons and therefore decided to carry the BART testers as a replacement for the dip-paddle. Once the BART products were in the marketplace there was a natural comparison with the agar spreadplate technique that still remains an industry standard. The technical personnel at Hach began to get a string of inquiries concerning the BART testers since these laboratories did comparisons between the BART tester data and the standard agar spreadplate techniques. The most common outcome was that these comparisons was that the BART testers generally reacted faster to the presence of the bacteria in the water than the spreadplate often to the extent that the agar spreadplate would remain negative while the parallel BART test would indicate a highly aggressive population of bacteria. The testing laboratories therefore automatically thought that it was the BART tester that was flawed since the agar spreadplate was negative and informed Hach on several occasions that the BART was a failure because it did not parallel the spreadplate. Hach had a standard reply which was for the BART to have reacted (often to a very aggressive bacterial presence) there were two options. The first option was that the BART tester was more sensitive than the agar spreadplate and the second option was that there had been a spontaneous generation of bacterial life in the otherwise sterile BART. The former option was accepted as being more reasonable. Hach has continued to market the BART testers but has not been aggressive since the BART introduces a new level of sensitivity beyond the ability of the entrenched agar spreadplate. One outcome was the realization that verification of the BART testers could not easily be achieved against the agar spreadplate technique.

There was from 1990 to 1996 some interest by the AWWA/APHA in having the BART testers included in the 19th edition of the Standard Methods for the Examination of Water and Wastewater with the suggestion that D. Roy Cullimore also rewrite the section on iron and sulfur bacteria. This could only be done by giving all of the formulae and details of the production of the BART and would have led to a

voiding of the patent. It was decided not to proceed but a contribution covering the sulfur bacteria was made to the AWWA publication on Nuisance Bacteria[xxxviii].

2.5 Semi-Qualitative Evaluation

There has been less attention paid to the examination of bacterial community structures as such but more to the ability to identify potential pathogenic or nuisance organisms. In the development of microbiology the emphasis has been increasingly placed on the recognition of very specific species (e.g., *Escherichia coli* strain 0157:H7) rather than on the composition of genera within the bacterial community functioning within specific environmental niches. In general classical microbiological approaches include attempts to identify species of potential concern using selective techniques ranging from culture medium selectivity, specific environmental conditions and the use of various forms of biochemical tags. Consequently the whole focus of modern microbiological investigations is centered on finding the very specific rather than the more general approach of examining for large communities. The IRB-BART tester differs from other test protocols in that a series of environments are created within the charged test vial. Because of these shifting dynamic environments there are many opportunities for a bacterial community to locate at site within which activity can commence. The types of community that can flourish are very much restricted by the selection of the culture medium that diffuses upward from the crystallized pellet in the floor of the tester.

2.5.1 Selection of IRB culture medium

Traditionally the culture media selected for the examination for iron bacteria were based around traditional agar plating techniques and the selective culture of specific groups within the IRB. In 1993, The Handbook of Microbiological Media[xxxix] included the iron bacteria isolation medium (for the isolation of iron bacteria) and the iron-oxidizing medium for the cultivation of iron and sulfur bacteria such as *Thiobacillus ferrooxidans.* Fe-amended heterotrophic plate count agar medium has been proposed to determine the iron related heterotrophic bacteria[xl]. A broad spectrum survey and comparison of IRB cultural techniques revealed a range of four simple field test at the presence /absence levels[xli] to a full review of the various culture media that had been employed prior to 1976 for the culture of iron bacteria[xlii]. Of the media for the differentiation of IRB, it was found that of the fourteen media evaluated for the sheath-forming IRB genus *Crenothrix* and the ribbon forming *Gallionella,* the highest recoveries were obtained obtained using Winogradsky's medium set at a pH of 7.3. It was subsequently found the using the liquid medium with a supplementation of ferric ammonium citrate further broadened the ability of the medium to detect the heterotrophic IRB. This medium became known as the WR (Winogradsky Regina medium) and the first description of an iron bacteria presence test was in 1980 where a positive was considered to be the formation of a yellow liquid or a brown scum at the fill line[xliii]. Detection of a positive reaction was extended in 1981 to include floating flakes[xliv]. Parallel studies at that time were with the membrane filter (MF) technique using the agar formulation of Winogradsky's original medium. Independent evaluation of the original iron bacteria presence test confirmed that the test was simple and convenient[xlv] For the original formulation of

the WR medium used in the IRB-BART from 1987 to 1993 the type of ferric ammonium citrate used was the "brown" form that had a much more variable consistency and physical form and no reliable chemical data sheets. In the early development of the IRB-BART this "brown" form was found to give lower precision than the "green" form. In consequence of this the formulation from 1993 used the "green" form exclusively with improved reliability. Final recognition of the WR formulation for IRB detection came from its inclusion as an effective medium when compared to other selective media for IRB[xlvi]. The WR using the "green" form has been the standard since 1993 and has been consistent in the activities and reactions observed under controlled conditions.

After the important selective nature of the WR medium as a vital component in the effectiveness of the IRB-BART, the next major factor is the development of a variety of micro-environments in a manner similar to the Winogradsky column[xlvii]. The net result commonly observed is that activities of different types occur within the IRB-BART tester. Reactions follow a common event pattern as first, secondary and final reactions. These are listed in the Table Four:

Table Four
Locations of IRB activity within the inner test vial of the IRB-BART when the iron related bacteria are present.

Position in IRB-BART	First reaction	Second reaction	Final reaction
Above ball		Slime ring	Coatings
Around ball equator	Foam		
On underside of ball	Gas bubbles		Coatings
Mid-point of column	Clouds / turbidity	Colored solutions	Blackening
Lower part of column		Gel formations	Blackening
Conical base	Dissolution of medium		Black or white deposits

There are therefore five major focus sites where significant activities can occur. Of the reactions listed above, coatings on the ball are ignored since these are often thin and occur after the major reactions have been observed, white deposits do occur in the conical base of the IRB-BART in some waters and there has not been an adequate understanding to determine the cause of this event and therefore it is not included in the established reaction patterns.

The number of reactions recognized and the form of recording these reactions has changed since the inception of IRB-BART testing in 1987. Early experiences found 22 reactions types that were categorized in 1988[xlviii] based upon all distinguishable forms that could be recognized[xlix]. This was reduced to nine reactions in 1989[l] and raised to ten[li] in 1993 with a further additional reaction (13) for the presence of mold growth. The reaction code was numeric and this caused confusion with the users of the IRB-BART. To correct this problem, the reactions were changed to a two-letter coded format with eight recognized and clearly distinguishable differences[lii]. This has been well accepted by the users.

2.6 Quality Management

Droycon Bioconcepts Inc. received registration for ISO 9001:2000 in August, 2001. The frame work for the receipt of the ISO registration was a quality management system for the production of the IRB-BART product and also the operation of the design, research and experimental development within the company. The various documents directly related to the IRB-BART testers are summarized below. It should be noted that the documents are all preceded by either: QI (for instructions), QF (for forms) and QP (for procedures). These forms are numbered and revision given.

2.6.1 Production of the Plastic Vials for the IRB-BART Product.

The plastic vials include five components: inner vial, outer vial, inner cap, outer cap and the floatation ball. There are a number of documents that are related to these activities. They are listed in the Table Five:

Table Five
ISO 9001 Documents Relating to the Plastic Vials

Document number	Title	Revision number	Pages
QI 20	Sterilizing inner vials	0	1
QI 21	Sterilizing outer vials	1	1
QI 22	Sterilizing containers	0	1
QI 36	QM for sterility of vials/containers	0	1

2.6.2 Manufacturing Procedures for the IRB-BART testers

Bart manufacture is covered for the general procedures in QP 7.5-1 (revision 1, 9 pages). Packaging and shipping and covered in QP 7.5-5 (revision 2, 7 pages) with the control of non-conformance in-process and final products is addressed in QP 8.3-2 (revision 2, 2 pages). Dispensing the IRB-BART testers is addressed in QI 21 (revision 1, 2 pages) and balling the IRB-BART inner vial is given in QI 50 (revision 0, 2 pages). Other documentation relevant to the specific manufacturing process is dealt with specifically for each individual IRB-BART tester product.

2.6.3 General Quality Management Relevant to the BART testers

Administrative aspects of the quality management that are relevant to the manufacture of the BART tester product is listed in the Table Six:

Table Six
ISO 9001 Documents Relating to the Production of IRB-BART Testers

Document	Title	Revision number	Pages
QSM 1	Quality System Manual	5	6
QP 4.2.3	Control of Documents	3	4
QP 4.2.4	Control of Records	3	2
QP 5.4.2	Quality Plan	2	2
QP 7.4.1	Selection of Approved Suppliers	1	2
QP 7.5-5	Packaging and Shipping Procedure	2	7
QP 7.6	Control of Measuring and Monitoring Devices	3	6
QP 8.4	Use of Statistical Techniques	1	2
QP 8.5	Corrective and Preventative Action	3	3
QI 210	Retail Packaging / Shipping	0	1
QI 211	Wholesale Packaging / Shipping	0	1
QI 301	Laminar Flow Check Work Instructions	0	1
QI 306	Analytical Balance Equipment Check Work Instructions	1	1
QI 311	Autoclave and Gas Sterilizer Check Work Instructions	1	1
QI 314	Calibrating Dispensing Equipment Check Work Instructions	0	2
QF 10	Customer Survey	1	1
QF 11	Management Review Minutes Form	2	1
QF 22	Training Evaluation - Trainee	0	1
QF 30	Retail Sales Order	2	1
QF 31	Wholesale Sales Order	1	1
QF 32	Customer Request	3	1
QF 33	Quotation	2	1
QF 54	Raw Materials Inventory Work Sheet	0	2
QF 55	BART Inventory Work Sheet	1	2
QF 57	Monthly Production Log Sheet	0	1
QF 61	Nonconformance Report	1	1

Additional forms and procedures are also more indirectly related to the production of the BART testers and these would be made available on request to the ISO Manager at (306) 585 1762.

2.7 Verification Process

All batches of BART testers go through a quality management evaluation prior to release. This includes verification that the batch meets with the standards established by DBI which is then entered into the appropriate QF batch log sheet. When the batch is accepted as of sufficient standard then a certificate of analysis is released as a QF

that is provided to the customer with each box of IRB-BART testers (9 full field IRB-BART testers with outer vials or 15 inner laboratory IRB-BART testers).

3 BART Verification

There are two stages in the verification of the IRB-BART testers. First, the IRB-BART tester should be able to generate acceptable data with good precision for the semi-quantitative and semi-qualitative examination of the water sample for the bacterial group targeted through the selection of the culture medium crystallized into the base of the inner BART test vial. Second, there is the form of the interaction between the indigenous microorganisms in the water sample being tested with the multiplicity of environments that are forming and changing within the water column. In dealing with the IRB-BART testers two levels of evaluation are employed:

- Verification level assures that the specific BART tester meets with the criteria established to allow the tester to perform as claimed.

In the claims for the IRB-BART tester verification refers to the ability of the specific instrument to detect the IRB as a group at the semi-quantitative and semi-qualitative levels.

3.1 IRB-BART, Summary

IRB is the shortened form of "Iron Related Bacteria" and embraces those bacteria that are able to accumulate and use iron in any form beyond the basic metabolic needs that is commonly experienced by all microbial cells. This iron may be accumulated in insoluble forms of ferric iron (under oxidative conditions) or dispersed in the soluble ferrous forms (under reductive conditions). Standard text books on the subject still commonly separate these two events as being "iron oxidizing bacteria" or "iron reducing bacteria". Experiences in studying the ecology of iron-rich environments and during the development and use of the IRB-BART testers at such sites is that the bacterial communities are commonly able to both oxidize and reduce the iron depending primarily upon the ORP in the local environment.

3.1.1 Verification

The IRB-BART is manufactured using the recipe (QF 109, revision 0, 1 page) and the effectiveness of the IRB-BART is assessed using the IRB batch log sheet (QF 109, revision 0, 1 page) before a certificate of analysis is issued (QF 139, revision 1, 1 page). The finished and certified IRB-BART tester is immediately sealed in an aluminum foil tear-down pouch to protect it from rehydration and the shelf life is established at three years when stored in this manner in a cool dry place.

The primary element in the differential selective activity of the IRB in the BART tester is the use of ferric ammonium citrate in the selective culture medium. This compound was first recognized as being differential for iron bacteria during studies conducted in the late nineteenth century. In an IRB-BART tester where there are IRB active, it has been observed that it is common to see a green gel-like diffusion front rising from the WR nutrient pellet crystallized on the floor. This green color would be the result of the

reduction of the ferric form (commonly shades of yellow, red to brown) to the ferrous form by the indigenous IRB in the water sample. The rate at which the indigenous IRB can cause this ferrous front to form and mobilize up the IRB-BART tester has been found to be variable and no clear semi-quantitative or semi-qualitative linkages could be determined. Another differential impact is achieved through the use of citrate as a major carbon source in the IRB-BART further limits the growth of non-IRB bacteria that are in the water sample as a part of the indigenous flora but various researches have shown citrate can be used by the IRB. Ammonium from this compound provides a readily assimilable form of nitrogen that can be used by a wide spectrum of bacteria and can stimulated the growth of the IRB. The form of the ferric ammonium citrate is critical and only the green variety is used in the production of the IRB-BART. In the early development of the IRB-BART from 1987 to 1993, the brown version was employed but found to give variable results on a batch to batch basis and its use was discontinued in favor of the green form that gave consistency in the reaction patterns that were not achieved to that time.

3.1.2 Verification of Claims, semi-quantitative

From the field experiences of using the IRB-BART and laboratory trials, the following table has been established linking the time lag (Table Seven) to the first activity/reaction to the aggressivity and populations of IRB in the water[liii]:

Table Seven
Relationship of Time Lag to Aggressivity and Population of IRB-BART

Time lag (days)	Aggressivity level	Population range cfu IRB/ml
0.05 – 4.0	High	>1,000,000 to 1,000
4.05 – 8.0	Medium	999 to 11
8.05 – 10	Low	10 or less

Where there is an IRB reaction observed after ten days it is considered to be background and not aggressive. IRB activity/reaction has been observed after time lags as long as 42 days.

3.1.3 Verification of Claims, semi-qualitative

The RPS emerging from the IRB-BART where aggressivity is detected can be complex because there are eight recognized reaction codes that can be observed and there are many different combinations. There are however a number of RPS combinations that do occur commonly and in a consistent manner from specific sampled water sources. The following table lists some of the common RPS found for waters (Table Eight) where aggressive IRB have been detected. Commonly the first activity/reaction is restricted to either:

- CL which is a clouded growth that commonly occurs in the middle of the water column at the forming redox front. This is the most common first

reaction when there are aerobic IRB dominating the bacterial community. The presence of CL therefore indicates primarily that aerobic IRB are dominant in the water sample.

- FO is the formation of a foam ring formed by interconnected bubbles of gas around the floating ball. This foam can last one to five days before dissipating. Its presence as the first reaction indicates that the IRB in the water sample are primarily anaerobic in their activity.

Table Eight
Major RPS Groups Observed in the IRB-BART tester application to Waters

RPS	Family Type	Interpretation
CL - GC	I	Dominant aerobic bacterial species are pseudomonads
CL - BG	II	Enteric bacteria dominate with species of *Enterobacter*
CL- BC - BR	III	Complex community of aerobic IRB
CL - RC	II	Enteric bacteria possibly dominated with species of *Citrobacter, Serratia* and *Klebsiella*
CL - BL	IV	Aerobic community includes pseudomonad and enteric bacteria
CL – FO – other codes	III	The community is dominated by aerobes with some anaerobes
FO - BL	V	Anaerobic community includes dominant enteric with some pseudomonad bacteria present
FO – BC - BR	V	Complex community of anaerobic IRB
FO – CL – other codes	V	The community is dominated by anaerobes with some aerobes

The common (85 to 90% of samples evaluated) first reaction observed is one of the above two clearly differentiating aerobic (CL) from anaerobic (FO) communities. The identification of the IRB bacterial communities is essentially achieved by the subsequent activities/reactions that are observed. While this has been documented in the literature[livlv], the later codes observed can be sequenced into the RPS and allow further identification of the nature of the IRB community. Some of the common RPS values observed in water are listed above. These RPS combinations of two or three codes are commonly observed within water and longer RPW commonly involve these strands with additional entries that can be interpreted as additional sub-sets in the identification process. If the RPS was CL - BC - BR indicating the presence of a complex community of aerobic IRB but if there were two additional codes then each of these could be interpreted in relation to the table above. As an example of this the RPS was different including two more reactions: CL- GC - BC - BR - BL (new codes underlined) then the original interpretation would be modified to "a complex community of aerobic IRB including dominant species of pseudomonads (GC) and also some enteric bacteria (BL)". This reflects that the pseudomonads were dominant as the second code reaction while the enteric bacteria were only present in low numbers since they did not dominate any previous reactions.

3.1.4 Verification of the IRB-BART tester

The IRB-BART tester employs a variety of iron-rich environments that are changing quickly as a result of the formation of a redox front and diffusion of the selective culture medium from the base cone of the inner test vial. In the testing procedure 15ml of the original water sample is used and so the ability exists to detect any significantly aggressive IRB that may be present in that sample. This is the only test system that creates a multiplicity of environments within the water sample being examined in a manner that can trigger the growth of a broad range of IRB. Prior to that time testing was limited to agar spreadplate and membrane filter techniques using selective media for IRB[lvi]. Microscopic and *in-situ* cultural determination of IRB were being developed[lvii]. Some background to the evaluation of iron bacteria is included in the Table Nine:

Table Nine
Historical Development of the Determination of Iron Bacteria in Water

Year	Topic	Reference
1919	Iron bacteria, a comprehensive overview	[lviii]
1945	Iron transformation in water	[lix]
1948	Review of the iron bacteria	[lx]
1952	Iron organisms	[lxi]
1958	Cultivation and classification of iron bacteria	[lxii]
1958	Staining iron bacteria	[lxiii]
1974	Quick culturing and control of iron bacteria	[lxiv]
1978*	Overview of iron bacteria in ground water	[lxv]

The selective culture medium described in the paper (*) is now in use for the IRB-BART. It is based on the WR medium (Winogradsky Regina medium) developed and reported on in this paper. This paper is now commonly cited as one of the first papers beginning to refocus the place for IRB particularly in ground water systems. Prior to that time, routine testing was performed using the traditional membrane filter and agar spread plate techniques but failed to detect, in many cases, even when there was clear direct and microscopic evidence that IRB were present.

The primary validation of the IRB-BART is that it is has been employed in investigations where it has consistently shown a greater number of positive detections with more precision than either of the other two techniques described above. The shortage of a technique to determine the IRB in the field and in the laboratory setting has been challenging in the industry and the American Water Works Association Research Foundation (AWWARF) recognized this shortcoming in funding Leggette, Brashears and Graham Inc (LBG) of Connecticut to undertake a study of the current water well rehabilitation techniques in use. With the support of the AWWARF, LBG selected the IRB-BART as being the only test that would allow them to measure the level of IRB activity in the ground water before and after treatments[lxvi]. DBI therefore became a part of the investigative team and

provided the IRB-BART for use during the investigation[lxvii]. In the period of time since 1986 there have been a number of events that form, it is claimed, a validation of the use of the IRB-BART tester for the determination of the aggressivity and consortial nature of the IRB in aquatic environments and more specifically ground waters. These events are listed chronologically in the Table Ten:

Table Ten
Major Publications Relating to the Use of the IRB-BART testers

Year	Topic	Reference
1993	Use of BART testers in ground water	[lxviii]
1994	Biological monitoring : Decision making and the IRB-BART	[lxix]
1998	The use of BART testers in wells rehabilitation	[lxx]
1999	The use of BART testers in control of biofouling	[lxxi]
2000	Use of BART testers in determining well biofouling	[lxxii]
2000	Identification of bacterial consortia using BART testers	[lxxiii]

A fuller detail of the publications relating to the development of the IRB-BART are listed below in chronological order are included in Table Eleven below:

Table Eleven
Historical Development and Verification of the IRB-BART

Year	Topic	Reference
1986	Concept developed by Roy Cullimore and George Alford during the AWRC symposium on biofouled aquifers	[lxxiv]
1987	Research concentrated on the IRB-BART. First use of the BART in well rehabilitation at the Grenada Dam, Mississippi by Mircon Consulting (Estevan) Ltd	[lxxv]
1988	Research concentrated on the SRB- and the SLYM-BART. BART detectors used to determine locations of biofouling in extraction wells at Stone Container Paper in Missoula, Montana	[lxxvi]
1988	BART testers (IRB and SRB) were used to detect biofouling and trigger rehabilitation treatments – still being used to present time for preventative maintenance	[lxxvii]
1988	IRB- BARTs used in the development of certification processes for Organic Farmers	[lxxviii]
1989	IRB-BART described in the Canadian Water Well Journal	[lxxix]
1989	Frequency of IRB in Canadian ground waters discussed	[lxxx]
1989	Layne –Western Company, Inc releases first description of the use of BARTs in well rehabilitation	[lxxxi]
1990	IRB-BART applications discussed at an International Conference on Microbiology in Civil Engineering	[lxxxii]

1990	U.S. Patent issued on the BART tester	lxxxiii
1990	Comparison of the use of the BART technology with the standard methods	lxxxiv
1990	The use of the IRB-BART testers in the determination of the treatment effectiveness of the rehabilitation of water well biofouling	lxxxv
1990	The use of the IRB-BART testers to determine the effectiveness of the treatment of biofouled water wells	lxxxvi
1990	IRB-BART testers systems described by Layne-Western Company Inc	lxxxvii
1990	IRB-BARTs are used by Ortech International, Missisauga, Ont to determine the effectiveness of in situ barriers to control BTEX	lxxxviii
1990	IRB-BARTs used in the evaluation of Biofouling of water wells in Newcastle, N.B	lxxxix
1992	IRB-BART testers were used on the examination of rusticles recovered from the RMS Titanic	xc
1993	First full description of the IRB-BART with protocol and interpretation methodologies	xci
1993	Atomic Energy of Canada uses the IRB-BART for field analyses at Pinawa, Manitoba URL	xcii
1993	Discussion of the use of the IRB-BART in an AWWA study of the evaluation and restoration of water supply wells	xciii
1994	First full BART interpretation and Reaction Chart	xciv
1996	IRB-BART tester recognized in a major book “Microbial Quality of Water Supply in Distribution Systems” by Edwin Geldreich	xcv
1996	Japanese version of the BART comparator chart prepared	xcvi
1997	Description of the use of the BART testers on the *RMS Titanic* in the 1996 Discovery Channel expedition	xcvii
1997	Hach Corporation includes the use of the BART testers on the RMS Titanic in 1996	xcviii
1997	IRB discovered on the RMS Titanic using the IRB-BART	xcix
1997	IRB-BART used by Canada Agriculture and Agri-Food to determine fouling problems in water wells in the Kneehill municipal district of Alberta	c
1997	Public advisory was released by PFRA including the IRB infestation of well in Kneehill M.D.	ci
1998	IRB-BART used in a joint project with PFRA to assess effectiveness of UAB treatment	cii
1999	IRB-BART methods discussed in “Iron and Manganese Removal Handbook published by the American Water Works Association	ciii

1999	Use of the IRB-BART discussed in Water Well Rehabilitation	civ
1999-2001	National Ground Water Association organizes a series of two-day workshops on water well rehabilitation including the use of the IRB-BART in Denver, 1999; Milwaukee, 2000, & Las Vegas, 2001	cv
2000	Expanded description of the IRB-BART and interpretation methodologies	cvi
2000	Determination of microbial composition of rusticles using the BART testers	cvii
2000	PFRA releases final phase report including the use of the IRB-BART	cviii
2000	U.S. Army Corp of Engineers release management engineering pamphlet on the rehabilitation of injection and extraction wells involving the IRB-BART	cix
2001	U.S. Army Corp of Engineers in final editorial stages of releasing an engineering pamphlet on the maintenance of injection and extraction wells at HTRW sites, the document is 120 pages and does include the use of the IRB-BART	cx
2001	Comprehensive evaluation of the BARTs to predict biofouling in porous media as a joint PFRA / DBI project	cxi

4 Summary of Claims for Verification of the IRB-BART

The following is a list of the major features that should allow for the environmental verification of the IRB-BART as a suitable technique for the detection, enrichment and enumeration of the iron related bacteria at the semi-quantitative and semi-qualitative level:

4.1 Definition of IRB

IRB for the purposes of the test shall be primarily classified as the iron related bacteria. The nature of the IRB-BART employing a vertical array of different and changing lateral environmental niches would allow the undiluted indigenous organisms to establish focal sites for metabolic activities and growth under suitable oxidative or reductive conditions. It is proposed that the nature of the IRB-BART provides an adequate amount of iron in both the oxidized (ferric) and the reduced (ferrous) form when there are IRB present.

4.2 Selective Culture Medium for the IRB

Winogradsky's Regina (WR) has been well recognized as a suitable culture medium for the enrichment of the IRB. In the early development of the IRB-BART it was found through practical experiential observations that the medium could be improved by the replacement of brown form of ferric ammonium citrate with the green form. Sodium thiosulfate was also added to reduce the impact of any residual

chlorine that may be in the water sample. The modified WR medium is presented as a crystallized pellet implanted from x10 strength concentrates.

4.3 Sample Management in the IRB-BART test

The water sample in the IRB-BART test is used directly without dilution. This means that the indigenous microorganisms is not impacted by dilution and that the technician conducting the test can apply 15ml of sample directly to the IRB-BART.

4.4 The Novel Format Created in the IRB-BART

A major feature of the patent is that an aspect ratio is created that causes the indigenous microorganisms in the water sample to focus at different micro-environmental sites that are created by the elevating diffusion gradient of the selective medium and the shifting reduction-oxidation gradient being created as any intrinsic oxygen in the IRB-BART test is consumed by the indigenous microorganisms.

4.5 Incubation of the IRB-BART

Through practice, it has been found that room temperature with a nominal optimum of between 21 and 23^{o}C is adequate to achieve a result within ten days. Room temperature was selected as a convenient temperature for testing where laboratory incubation facilities do not exist. To incubate the charged IRB-BART it should be kept in a location where the temperature is not likely to radically fluctuate and away from direct sunlight. The IRB-BART should not be shaken during observations for the detection of positive signals since this would disturb the formation of the oxidation-reduction and the nutrient gradients and could also introduce oxygen into the incubating sample.

4.6 Incubation times for the IRB-BART tester

The recommended time frame for the incubation of the IRB-BART is ten days. Under some circumstances positive detections may continue until the 14th day but these would be considered as “background”.

4.7 Determination of a Positive Activity for the IRB-BART tester

IRB activity is recognized by the formation of a variety of reactions that can be used to form a reaction pattern signature through which the IRB can be categorized as belonging to one of five major IRB families. Since there are no dilution series, the observation is of the original water sample in the IRB-BART tester and so there is a minimum of technician time employed.

4.8 Semi-Quantitative Evaluation, Aggressivity

Aggressivity of the IRB in the water sample being tested is determined by the time lag during incubation up and to the time that the first activity was recorded. This time lag can be used to determine the aggressivity. High aggressivity would mean that the time lag would be at 4days or less. Medium aggressivity would have a time lag of between 4 and 8 days while a low would have a time lag of between 8 and 10

days. If the time lag is greater than 10 days, this would mean that the level of IRB would be "background" and therefore not significant.

4.9 Semi-Qualitative Evaluation, IRB consortial identification

Because there are three distinct activities that can be observed in the IRB-BART tester, it is possible to undertake a semi-qualitative identification of the bacterial consortia associated with the IRB. This is described in Table Eight with five possible consortial family combinations.

4.10 Confirmation of the Presence of IRB in the Positive IRB-BART tester

The confirmatory method to confirm the presence of IRB could be achieved because the IRB-BART acts as an enrichment medium that allows easier confirmatory studies for the IRB to be performed in the laboratory using the standard recognized methods.

5, Primary Claim

The IRB-BART generates, when charged with a water sample, a sufficient diversity of environments that will encourage the determination of observable activities of the IRB within the water sample being tested. From experiences to-date the IRB-BART tester appears to be superior to any other field-applicable testing system due to the broad scope of IRB that can be recovered using this tester. It is proposed that the methodologies and technical information relating to the IRB-BART tester are sufficient for the verification of the Biodetector as a suitable system for the detection of IRB in water-based samples. These would be subject to the following limitations:

1. The limits of detection for the IRB in a given water sample would be 67cells/l.
2. Any water sample taken for testing using the IRB-BART tester would have to be collected following the protocols established for the collection of a water sample for microbiological analysis. Transportation and storage of the sample should similarly follow the standard guidelines practiced for sample handling prior to the initiation of microbiological examination. These should include hygienic aseptic handling, the use of sterile sample containers and minimizing the storage time to less than four hours at room temperature or twenty four hours when cooled to refrigeration temperatures.
3. The IRB-BART can be used for both field and laboratories based investigations and generate similar data with respect to time lag and reaction patterns where a sample is split and incubated under similar conditions in field and laboratory settings.
4. While the IRB-BART technology commonly operates at ambient room temperatures there is the ability for the testers to be used at incubation temperatures ranging from +1 to +55^{o}C under exceptional circumstances.

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