ENVIRONMENTAL TECHNOLOGY VERIFICATION

OF THE

SRB-BART™ TESTER

FOR THE

DETECTION AND EVALUATION

OF

SULFATE REDUCING BACTERIA IN WATER

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

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1, Introduction

This document relates to the submission to ETV Canada Inc of materials in support of the verification of the SRB-BART^{TM} tester by that agency. The name of the trademarked product (SRB-BART) stands for biological activity reaction test and, for the purposes of this verification, is limited to the sulfate reducing bacteria (SRB) that cause in any manner corrosive and nuisance bacteriological activities often 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. It is intended to demonstrate the claims relating to the verification of the SRB-BART tester on a scientific and technical basis in which both internal and independent information will be presented. In addition the claims will also address the relative convenience and confidence of the test in comparison with existing laboratory and field tests.

2 Concepts

The concept involved in the SRB-BART tester is presented in seven parts. These may be subdivided into the historical development and selection of an instrument that would detect SRB corrosive and 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 SRB leading to the current level of understanding is summarized in the following table.

Table One
Historical Development of Understandings on the Sulfate Reducing Bacteria

Year	Author	Topic
1895	Beijerinck[i]	Described a sulfate reducing *Spirillum*
1903	Van Delden[ii]	First pure culture isolated
1930	Baars[iii]	Fatty acids identified as substrates for growth
1936	Kluyver and van Neil[iv]	Classification of sulfate reducing bacteria
1959	Postgate[v]	Review on sulfate reducing bacteria
1965	Postgate[vi]	Review of the sulfate reducing bacteria
1966	Postgate and Campbell[vii]	Classification of *Desulfovibrio*
1981	Pfenning, Widdel and Trüper[viii]	Classification of Dissimilatory Sulfate-Reducing Bacteria

Unlike the IRB, the sulfate reducing bacteria (SRB) are a closely definable group of bacteria. The nature of this group was defined by Madigan, Martinenko and Parker[ix] in 1997. SRB have long been known to be involved in biocorrosive processes due, in part, to the generation of hydrogen sulfide that then initiates the electrolytic corrosion of metals[x].

The SRB form an integral part of the group referred to as "Sulfur Bacteria" and are considered to be nuisance bacteria in a number of ways[xi]. Included in the sulfur bacteria group are:

- Sulfate Reducing Bacteria
- Sulfur Oxidizing Bacteria
- Sulfur Reducing Bacteria

The SRB-BART specifically detects the first group that is capable of generating hydrogen sulfide from the reduction of sulfate. A number of problems are related

to the generation of H_2S. There are three major events[xii] that can result from the presence of this gas:

- Odor generation resembling rotten eggs
- Electrolytic corrosive processes
- Generation of black iron sulfides

In economic terms, the SRB ranks as one of the most severe nuisance bacterial groups because it has been linked to the corrosion of steel structures such as pipelines, cooling towers, water treatment and synthetic chemical production plants. Considerable effort is expended to alleviate the challenges by cathodic protection that carries a high energy cost while failure carries a high capitalization cost. At this time the SRB-BART has generated the most demand due its adoption by Canadian companies in the oil and gas sector mainly to evaluate the effectiveness of management practices in the control of recognized corrosion risks.

Much of the development of technologies in microbiology in the twentieth century focused on species of recognized cultivable pathogens and it was not until the middle years that attention began to be paid to the non-pathogenic nuisance bacteria. As a result of this emphasis, 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 (between 1930 and 1960) with a slow resurgence towards the end of the twentieth century when sustainability and environmental-risk impacts became significant concerns. The rapid growth of molecular and genetic tools in the last two decades has meant that the specificity for identifying 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 SRB activities causing specific nuisance events. Today more attention is being paid to the identification of bacterial communities (consortia) as such rather than the component cultivable strains. The consortial composition for the SRB was defined for the SRB-BART BT (black top) and BB (black base) reactions was defined in 2000[xiii].

The obsession with micro-cellular studies has denied to some extent the understanding 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[xiv] which included 37 references of which 9 related to the SRB. 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 three[xv] in that manual was devoted to sulfate reducing bacteria as a major group in the nuisance sulfur bacteria covered in that chapter. In 1992 there was a full evaluation of the contemporary perspectives for the SRB[xvi]. Today considerable attention is being paid to the application of 16S rRNA sequence information with respect to phylogenetic studies to aid in the characterization of the SRB[xvii]. These initiatives provide a means of examining the structure and diversity of the SRB communities that form a coherent assemblage[xviii]. In ecological studies, it was noted that in areas with a high H_2S production reflected in as much as 30% of the total rRNA extracted being SRB rRNA. Phylogenetic analysis was employed using a polyphasic approach to the speciation of *Desulfovibrio* in oligotrophic oxic environments[xix]. Consortial relationships were found between strain B3 and *Aquabacterium commune* growing within a common biofilm.

A further component in the SRB that has potential value in the identification is the cytochrome c3 since this one specifically found in the complex electron transfer chain of SRB[xx]. This cytochrome can be characterized by size, the tetraheam nature, the bishistidinyl ligation of the heam iron atoms that are distinct at low redox potentials.

These initiatives to determine with some precision the nature of the SRB in water and soil samples but the cost is increased particularly when cultivation is used prior to the biochemical determination of the rRNA. The premise behind the SRB-BART is to be able to effectively detect the presence of the major communities of SRB and their level of aggressivity in the water sample. Many of the basic concepts are as relevant to the SRB-BART as they are to the other BART testers.

2.1.1 **Development of the SRB-BART tester**

For the SRB-BART tester, a search began in 1986 with the development of the invention that became established in the patented BART tester. Through the precedent development of the concepts leading to the IRB-BART tester, an understanding was generated of the complex inter-relationships that exist in biofouled situations including colonization mechanisms[xxi], formation of biofilms[xxii] and the challenge of monitoring methods[xxiii]. It became clear at the symposium and at a precedent think tank[xxiv] that the evaluation of microbes in ground water is made that more changing because of the following factors:

- The SRB were recognized to flourish in reductive environments and that these bacteria could integrate in communities forming sessile or suspended biofilms
- Much of SRB activity is likely to occur within the biofilms attached to surfaces and not to such an extent in suspended biofilms in the water. This would infer that a water sample may not be representative of the activity of the SRB associated with the biofilms growing attached to the surface and not sheering in any manner. This may be particularly the case where the SRB are entrenched into the deeper more reductive regions of the biofilm.
- Much of the microbial activity in the water is of a biocolloidal nature[xxv] in which the microbes are contained within a polymeric matrix of bound water.

For the SRB, given their anaerobic nature, to thrive within these biocolloids they would need to be shielded from the oxidative environment through the activities of other bacteria that are able to function aerobically. This concept is only now beginning to change in very fundamental ways the manner in which water can be viewed

- Few microorganisms are planktonic and freely suspended with unbound (free) liquid water and probably no SRB unless the environment itself is highly reductive
- A water sample may not reflect SRB that are retained within biofilms attached to surfaces if none are released as large reductive biocolloids into the sample
- Examination for SRB may be compromised by the biocolloids restricting the determination of SRB activities within the test procedure.

Prototype BART testing for the SRB was begun in 1987 initially using 10ml of water sample. To generate reductive conditions to encourage SRB activities, the early SRB-BART testers had mineral oil added (1.0 reducing to 0.2ml). This was done in order to reduce oxygen penetration through the glycerol acting as a floating barrier to the downwards diffusion of oxygen around the float ball. Poor precision continued until the water sample volume used for testing was increased to 15ml. However, the only positive detection of the SRB occurred when there was a blackening in the base of the test vial. This was designated a black base (BB) reaction. Between 1989 and 1995, the mineral oil added was progressively cut to 0.1 and then to 0.05ml. When the mineral oil was reduced to this extent it formed a thin film over the water sample reducing but not eliminating oxygen transfer. It was noticed that a reaction began to be observed in which there was a distinct blackening around the float ball (lower hemisphere) and this was designated a black top (BT) reaction. In the subsequent years to the present time, it has been found that the reaction first observed in positive SRB detection is either the BB or the BT and that on some occasions this is followed a blackening of all the contents (BA). On the basis of the SRB-BART test results used in various trials since 1995, the following conclusions can be made concerning these reactions:

1. BB reaction is usually associated with a condition where the water sample has been taken from a highly reductive condition
2. BT reaction commonly occurs under more eutrophic oxidative conditions when there is extensive aerobic biofilms occurring. Here it is common to find highly aggressive heterotrophic and slime forming bacteria also present.
3. BB – BA RPS signifies that the dense anaerobic SRB are also (under the incubation conditions in the SRB-BART) able to adapt to more oxidative conditions and move up to cause blackening of the float ball.
4. BT-BA RPS signifies that there are covert SRB surviving within aerobic bacterial activity around the float ball and that they are able to adapt to colonize down the full length of the sampled water column to cause a generalized blackening.

The relative occurrence of the BB or the BB reaction in an SRB-BART can therefore indicate some information concerning the nature of the SRB growing at a site. For example, joint studies conducted at the Three Hills M.D. Alberta with Canada Agriculture and Agri-Food (PFRA branch) involving 134 wells showed that[xxvi]:

15% water quality problems
10% water production problems
49% had both quality and production problems

of the wells tested using the SRB-BART, 67% contained highly aggressive SRB mostly showing the BT – BA RPS with a further 25% moderately aggressive. The SRB appeared to dominate the bacterial communities associated with the fouling since for the same samples only 17% of the heterotrophic aerobic bacteria and 9% of the iron related bacteria were highly aggressive. Under these conditions, the biofouling of the water wells was strongly related to the SRB growing in a mixed aerobic microbial community under moderately oxidative conditions.
Parallel studies were conducted independently by the PFRA at the rural municipality of Mount Hope paralleling the study at Three Hills M.D.[xxvii]. Here the study included 193 wells of which 131 were active with 62% of these reporting symptoms that could be related to biofouling. Dominant was the IRB with 73% of the wells tested being found to be highly aggressive. The SRB were highly aggressive in 34.5% and Heterotrophs in 42% of the samples. For the SRB there was an approximately equal mixture of BT and BB as the first reactions signifying positivity.

Comparison of the data together with some original field studies undertaken by DBI indicate the potential for the SRB (BT reaction) to be related to underlying gas and oil fields such as the Viking field that lies partly under the Three Hills M.D. while there is no evidence of oil or gas fields within 150 kilometers of Mount Hope R.D.[xxviii]. In this study using time lag comparisons (shorter the time lag in days, the greater the aggressivity), the SRB were found to be much more aggressive (3.5±2.2days) at Three Hill M.D than at Mount Hope R.D. (6.7±2.1days). For the reactions observed for the SRB at Three Hills was 45% BT, 48% BA and only 7% BB (129 samples) while for Mount Hope the corresponding percentiles were 40%, 24% and 4% while 33% failed to detect any SRB activity.

The reaction patterns for the SRB-BART were stabilized at BB, BT and BA with BA as a terminal reaction in 1995. There are some other reactions and activities[xxix] associable with the anaerobic heterotrophic bacteria that were originally also included. These were taken out since the users tended to view those reactions as being positive for the presence of SRB. These reactions usually took the form of dense colorless or grey colloidal (gel-like) masses usually floating in the middle and/or lower parts of the liquid column. To prevent confusion these reactions are now ignored since they are not relevant to the detection of SRB.

The SRB-BART was therefore found to be able to generate a stable pattern of reactions and activities with precision. The term "reaction" was taken to relate to the manner in which the indigenous SRB within the sample generated either a blackening condition around the base of the SRB-BART during incubation as a BB reaction, as black specks gradually spreading to cover the lower hemisphere of the floating ball (BT), or as blackening extending to the whole length of the incubating sample (BA). The term "activity" was taken to relate to the time lag before the reactions became observable in the SRB-BART tester. The hypothesis was that the shorter the time lag to the observation of a reaction then the more active would be the microbial population in the sample under the conditions of the test. The first reports on the use of the SRB-BART were reported in 1990[xxx] [xxxi] [xxxii] [xxxiii]. At that time the BART was referred to as biological activity test (BAT) but this name could not be used as the trademark while BART could.

Between 1988 and 1992, the concepts originally applied to the IRB-BART were adapted to increase the range of potential BART testers including the SRB-BART. 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	SRB-BART	Retailed from 1989
1987 - 1990	Sulfate Reducing Bacteria	SRB-BART	Retailed from 1989
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

2.2 Functional Approach Selection

The basic premise in all of the BART testers that are being presented for verification is listed in the BART verification 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[xxxiv]. The use of the SRB-BART was described by Smith[xxxv] in 1995 and Mansuy[xxxvi] 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[xxxvii]. Aspects of the relationship of the

reaction patterns for the different BART testers 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)[xxxviii]. The concepts for which claims can be made that are also universal to all of the BART testers besides are the SRB-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 and the 0.05ml of mineral oil effectively leaves a headspace volume of air of 12.5ml including 2.5ml 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 SRB-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 SRB-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 color encoded cap (black) showing that it is an SRB-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 SRB-BART tests.

It is claimed that the use of 15ml gives an adequate volume of water to entrap a sufficient a range of SRB occurring in biocolloids, sloughed suspended biofilm materials and in the planktonic form to ensure that community will thrive

within the SRB-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 SRB-BART testers. For the field application of the SRB-BART testers then a cautionary note would be included that where the SRB-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 SRB-BART testers with water sample being proposed for verification in this document are:

- That 15ml of water sample is added to the SRB-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 SRB-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 SRB-BART tester.
- The maximum tolerance for error for filling the SRB-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 SRB-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 2.2.4) 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 380.2mm^2; this reduces the area for oxygen diffusion at the throat between the ball equator and the wall of the SRB-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 sloped 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 a site 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.

It should be noted that the SRB-BART includes in the production of the tester the addition of 0.05ml sterile mineral oil which floats up on the water sample to form a floating film that restricts oxygen entry into the water. This allows the reductive conditions to develop more quickly during incubation and encourage faster development of the SRB.

Claims relating specifically to the float ball of the SRB-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. Gases can collect and 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 SRB-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 a greater variety of environments than are usually presented in microbiological test procedures.
- The addition of sterile mineral oil causes conditions to be generated more rapidly for the detection of SRB activities. This oil is dispensed after the medium has been crystallized. By doing this the oil soaks into the crystallized medium. There has been no evidence of the mineral oil subsequently being released until the water sample is added.

2.2.3 Generation of an Aspect Ratio

One major claim relating to the patented SRB-BART system is the creation by the mineral oil film at the air: water interface in which there is a floating ball as an air-intercedent device. This acts to restrict oxygen entry to hasten the formation of a reduction-oxidation gradient once there is any significant microbial respiration within the water sample being tested using the SRB-BART. The aspect ratio, as applied to the 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 SRB-BART tester without a float ball then aspect would be exposed water surface area: volume which would be 1: 3.95 which would mean that for every square centimeter of surface the oxygen could diffuse through there would be almost four ml (cm^3) of 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 SRB-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 SRB-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 SRB-BART test calculated from the lateral equator of the ball is therefore increased from 1: 3.95 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 SRB-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 tester and the equatorial region of the ball) was 125.7mm^2 which would have been 70.3% larger than the new SRB-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 SRB-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 SRB-BART test vial.

Claims relating to the aspect ratio of the SRB-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 SRB-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 SRB-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 microbes thus allowing a broader spectrum of organisms to become active and flourish.

2.2.4 Capping the SRB-BART tester

The SRB-BART tester vial is capped with a 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, and in particular hydrogen sulfide, arising from the microbial activities escaping into the atmosphere. Once screwed down the cap provides a water-tight seal in the event that the SRB-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 SRB-BART tester is the ability to select the type of bacterial community that could be detected. This is achieved using the Medium C for Sulfate Reducers (Postgate's Medium C)[xxxix] selective culture medium. There were two modifications to the original medium to improve the sensitivity of the test. First sodium hypochlorite ($Na_2S_2O_5.5H_2O$) was added to ensure the neutralization of any residual chlorine that may be in the water sample. Second the sodium

sulfate was replaced by calcium sulfate since there was a concern that the sodium ion may be inhibitory to some salt-sensitive strains of SRB. Once dried, the medium is moderately hydrophilic and can under highly humid conditions take up water slowly. To prevent this all SRB-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 SRB-BART is described in section 2.3 and detailed in Appendix One as a part of the quality management procedures.

The general effects of adding the water sample into a SRB-BART tester on the selective culture medium in the base. The following is a list of the normal events that follow the addition of the water sample. It should be noted that once the water sample has been added and the capped screwed firmly down then the SRB-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 the off-white crystallized coating of medium in the conical base of the SRB-BART. No obvious colored diffusion front normally generates.
- Event two, the water in the SRB-BART tester a diffusion front does form but is difficult to see rising up the water column in the SRB-BART test vial. Event three, the diffusion fronts dissipates to leave a clear solution.
- Event three, a thin film of mineral oil can sometimes be seen to have formed at the air: water interface around the floating ball.

The indigenous microbial population, when activated by the selective nutrient diffusion front, triggers activities and reactions that will then interfere with the manner in which these events occur. For the SRB-BART tester the manner of growth follows two primary patterns followed by one final reaction form. These are listed in Table Three

Table Three
Locations and Form of Primary and Final Reaction Forms in the SRB-BART

Reaction	Code	Descriptor
Primary	BB	Blackening in a circular manner outwards inwards creeping over the crystallized medium interface with the plastic floor of the test vial
Primary	BT	Black granular-lake structures form over the lower hemisphere of the floating ball and gradually spread to a generalized blackening
Final	BA	Lower walls of the test vial begin to blacken until visibility through the test vial is significantly disrupted

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

- That the specificity of a given SRB-BART for particular bacterial communities is primarily controlled by the election of the Postgate's Medium C selective culture medium crystallized into the conical floor of the SRB-BART tester vial. This medium was modified to reduce the risk of inhibition of sodium-sensitive SRB by replace sodium sulfate with calcium sulfate. A second modification was the addition of sodium thiosulfate to neutralize any residual chlorine that may be a water sample. This improved the recovery of SRB from waters that had been subjected to chlorine disinfection. This addition of thiosulfate would also provide a secondary source of sulfate for reduction to H_2S by the SRB.
- The reactions and activities observed in the SRB-BART tester may be interpreted in a semi-quantitative and semi-qualitative manner to provide information on the size and form of the SRB 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 SRB-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 SRB-BART tester. In a condition where there is no detectable SRB activity then the water sample may not, when placed in the SRB-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 and thus encourage the growth of the anaerobic SRB. 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[xl].

Claims relating to the generation of an ORP gradient in the SRB-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 a SRB-BART tester where there is biological activity that reduces the oxygen concentration in the water column.
- Specific SRB communities targeted by the SRB-BART test are likely to locate in a particular and characteristic manner at two sites along the vertical profile in the SRB-BART test in response to the establishment of the ORP gradients (see also Table Three).
- That a unique feature in the SRB-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 SRB to be recognized by activity at two specific sites followed on occasion by a more generalized reaction along the majority of the vertical length of the water column of the SRB-BART tester.

2.2.7 Incubation Conditions for the Testing Period

There is a considerable concern about the incubation temperatures at which the SRB-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[xli]. 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 SRB-BART tester it is normally recommended that tests be performed at controlled room temperature that can normally be expected in to have a mean of 22^{o}C. This temperature is adequate to allow the activity of psychrotrophic and mesophilic but not the psychrophilic SRB that normally would not be active at above 18^{o}C. There is clearly a trade off in selecting a wide incubation temperature range for the SRB-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 from the same environmental site. If the water samples were taken from sites having a temperature of below 8^{o}C then consideration should be given to running duplicate SRB-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 SRB while the room temperature would indicate the activity of the psychrotrophs and the mesophiles. Under circumstances where very hot water sites are being evaluated with ambient temperatures of between 50 and 65^{o}C then the incubation temperature can be set at 54±1^{o}C to determine the aggressivity and nature of the SRB in the sample.

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

- For field testing using the SRB-BART testers, it is recommended that testing be conducted at normal room temperature (ranging from 19 to 26^{o}C) with a mean of 22^{o}C.
- For testing when the original ambient temperature of the water sample when collected was at less than 8^{o}C using the SRB-BART testers, it is recommended that SRB-BART testers should be incubated at 8^{o}C
- For testing when the original ambient temperature of the water sample when collected was over the range from 50 to 65^{o}C using the SRB-BART testers then it is recommended that SRB-BART testers be incubated at 54^{o}C
- No incubation studies showed are conducted at temperatures exceeding 58^{o}C since the integrity of the SRB-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 SRB-BART testers. During the incubation, commonly at daily intervals, the SRB-BART testers are inspected visually for any activity that could be associated with the targeted bacterial activity. Inspection should involve the following stages listed in Table Four.

Table Four
Sequence for Observing Reactions in the SRB-BART

Step	Focal Indicator	Interpretation
One	Observe the side walls of the SRB-BART for blackening on the walls of the test vial	BA
Two	Examine the lower hemisphere of the floating ball for signs of blackening	BT
Three	Gently lift up the SRB-BART and examine the underside of the conical base of the test vial for the formation of a coherent circular zone of blackening around the center spike in the test vial	BB

These reactions are also discussed in detail in sections 2.4 and 3.1.3. Over the incubation temperature mean of 22^{o}C it is common for all of the reactions to be observed in the SRB-BART tester by the tenth day. All reactions observed that are relevant to the detection of a positive reaction in the SRB-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 SRB-BART testers there will only be a sequence of reactions going from BB or BT to BA. Where the BA is the first reaction observed then no more reactions (i.e., BT or BB) will be observed. In very aggressive SRB samples it is common for the BA reaction to occur so quickly that either the BT or BB is not observed.

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 SRB-BART tester as being typical of one of the target bacterial groups being determined, the time at which this occurs since the start of the test gives an indication of the level of activity (aggressivity / population). All of the information relating to the SRB-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 at which the SRB-BART tester is first charged should be recorded by calendar date and hour using the twenty four hour clock and incubation should begin immediately. When the first positive reaction is observed it should be recorded on the standard field BART tester data entry sheet by entering

the time (to the nearest hour) and the observed reaction code from the three possible options. The time lag is the difference between the time at which the 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 if the first reaction was observed at 16:30 on the day following the start up at 08:30 of the SRB-BART tester then the time difference would be 1day and 10 hours which would be shown as 1.4days. The targeted SRB groups are considered to be absent from the when no reaction has been observed by day ten.

2.2.10 Semi-Quantitative Evaluation Mechanisms, Aggressivity

The aggressivity of the targeted SRB 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 [xlii] generating too narrow a spectrum of colonial growth from the targeted bacterial community. This is a particular problem for the SRB that are considered to be strictly anaerobic and therefore not capable of growing in an oxygen-rich environment. Consequently enumerative techniques have had to exclude oxygen by replacement of air with suitable neutral or reductive gases or applying a barrier such as a 15 to 20mm plug of agar to reduce oxygen entry[xliii]
The use of aggressivity is claimed to be valid since it reflects the ability of the intrinsic indigenous SRB in the water sample to grow in the SRB-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 numbers. 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 SRB-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 Five.

Table Five
Definition of Aggressivity and its Relationship to Time Lag
For the SRB-BART tester

Aggressivity	Definition	Relationship to Time Lag
High <6.1 days	There is an observed reaction that occurs quickly after the start of the SRB-BART tester being incubated indicating that there is either a very large or very active population of SRB	Shorter than the first critical time lag marker event
Medium 6.1 to ≤8.0 days	There is a significant delay before the recognition of the first reaction after the start of the SRB-BART tester being incubated indicating that there is either a moderately active or modest population SRB	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 SRB-BART tester being incubated indicating that there is either a very small population of SRB or that the SRB have a low level of activity and are not able to become very active in the SRB-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 the targeted SRB group in the water sample were not able to be active in the SRB-BART test due to either too small a population threshold or that the SRB were 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 SRB-BART tester when it is considered that there can no longer be a highly aggressive community in the water sample being tested. The second critical marker event is that time interval from the start of the incubation of the SRB-BART tester when it is considered that there can no longer be a moderately aggressive SRB community in the water sample being tested. The third critical marker event is that time interval from the start of the incubation of the SRB-BART tester when it is considered that there can no longer be an SRB 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 SRB-BART tester being proposed for verification in this document are:

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

2.2.11 Semi-Qualitative Evaluation Mechanisms, Reaction Pattern Signature

While the SRB-BART testers are capable of assessment of the activity level for the targeted SRB community 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 SRB by the sequence of reactions that are recognized during the testing period. Using the SRB-BART tester data entry sheet, the chronological sequence with which the reactions is obtained by the recognized coded reactions (i.e., BB, BT and BA) listed on the sheet. This string of codes can easily be converted into a reaction pattern signature (RPS) that may then be used to identify the nature and composition of the detected SRB 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 the number of discrete reactions that occurred in the record.
- Where more than a single reaction occurs on the same day of incubation then a comma (,) is inserted between the concurrent codes. This indicates that both reactions occurred essentially at the same time.

There is a limited number of possible RPS for the SRB due to the limited number of codes and the sequence that they occur in (see Table Four).

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

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

2.2.12 Record Keeping and Preliminary Interpretation

There are three levels of record keeping for the SRB-BART tester that is generic to all of the BART testers being proposed for verification. These include:

- General 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.
- Specific SRB-BART tester data entry sheet
  The specific SRB-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.

  Note: both the general and the SRB-BART data entry sheets 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.
- SRB-BART data interpretation chart
  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 SRB-BART testers. The center of the chart provides a conversion from log to arithmetic population. Only these 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 SRB-BART testers being proposed for verification in this document are:

- All information relating to the application of the SRB-BART testers in the field should be recorded on the standard BART tester data entry sheet
- All information relating to the application of the SRB-BART testers in the laboratory should be recorded on the standard data entry sheet
- Any interpretation of the RPS and time lag data should performed using the BART data interpretation 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 SRB-BART tester

From the beginning of the development of the SRB-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 ratio, an unstable condition was created when the test vial was knocked in any manner. This was particularly a problem 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 bases to the vial walls. 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 SRB-BART testers charged with water samples. This created a severe hygiene risk (due to cultured microbes escaping from the compromised SRB-BART tester).
- One almost inevitable bi-product of the growth of microbes in the SRB-BART tester is the generation of the hydrogen sulfide odors 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 SRB-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 SRB-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 place the SRB-BART tester inside an outer 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 firmly in a central and locked position. The outer BART tester vial provides the following advantages: (1) all odors generated by the SRB-BART tester during incubation are retained; (2) any leakages from the inner SRB-BART tester vial are contained by the outer vial; (3) the inner SRB-BART tester vial is protected from damage by sudden physical stresses; and (4) the outer SRB-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 a new 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) elected 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 SRB-BART test vial is pulled up and out of the outer SRB-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 SRB-BART tester vial and seals off the environment inside the 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 SRB-BART tester during incubation in the field.

2.2.14 Security in the Application of the Laboratory SRB-BART tester (inner test vial format).

In the analytical laboratory setting, there is a desire to conduct the SRB-BART test as economically as possible. The laboratory SRB-BART testers are provided in a minimal form for the completion of the test. Here the inner vial of the SRB-BART tester is provided without the protective outer BART tester 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 SRB-BART testers are: (1) the SRB-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 SRB-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 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 SRB-BART tester is suspended then the hole 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 SRB-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 SRB-BART testers being proposed for verification in this document are:

- That the field SRB-BART tester provides a secure method for the undertaking of a microbiological investigation of the activity and form of the SRB. The risks of damage to the tester, of leakages of odorous materials or microbes from the containment in the inner SRB-BART test vial are reduced to negligible proportions.
- That the field SRB-BART tester provides a convenient field testing technique that can be set up at remote locations and provide information at-site.
- That the field SRB-BART testers can be returned to a laboratory should confirmatory microbiological studies need to be undertaken on any microbial cultures generated and observed during the testing period.
- That the laboratory SRB-BART tester provides a convenient technique for the determination of the SRB community in an economical manner.

2.2.15 Disposal Issues for the SRB-BART

There is naturally a concern that spent SRB-BART testers are disposed 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 and so pasteurize the contents.

Some investigators may dispose of the spent SRB-BART testers by unscrewing of the caps and tipping the contents into a pail of bleach solution which is kept overnight before disposal by pouring down a drain. This technique is not recommended.

2.3 SRB 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 by their presence, growth or activity cause a nuisance condition to be generated. This in turn 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 SRB-BART testers the definition is restricted to the nuisance bacteria that have an indirect impact.

For the SRB one of the major products of growth is the generation of hydrogen sulfide. The World Health Organization recognizes this and recognizes a number of sources[xliv] for this gas:

> "Hydrogen sulfide is one of the principal compounds in the natural cycle of sulfur in the environment… It occurs in volcanic gases and is produced by bacterial action during the decay of both plant and animal protein… it can also be produced through the direct reduction of sulfate"
>
> "Odor threshold for hydrogen sulfide has been reported to range from 0.0008 to 0.2 mg/m^3 (0.0005 – 0.13ppm)… Task group considered that 0.008mg/m^3 (0.005ppm) averaged over 30 minutes should not produce an odor nuisance…earliest toxic response appears to be eye irritation which has been reported to occur at 16-32 mg/m^3 (10.5 – 21.0ppm"
>
> "Ambient air level of hydrogen sulfide to be 0.0003 mg/mg^3 (0.0002ppm)… numerous fatalities occurred indicating that exposure levels were most likely in excess of 1500 – 3000 mg/m^3 (1000 to 3000ppm)"

For severe symptoms to be experienced it is generally thought these arise from catastrophic natural geological events or industrial plant failures. For the biogenic production of hydrogen sulfide, it is mainly considered that comes from bacterial action[xlv]. The major sources are sulfate and sulfur-containing amino acids in the biomass (listed as plant and animal protein). Sulfate forms the pivotal form of sulfur that can either be assimilated by living organisms or reduced. Various bacterial groups are able to shift the sulfur between the S^o, SO_2 and the SO_4 states where it can be reduced to hydrogen sulfide by the SRB. There is a general consensus that most of the H_2S generated in the environment biologically arises from the activities of SRB. Little attention is however paid to the proteolytic degradation of the sulfur-containing amino acids that can also generate H_2S along with ammonium as terminal products of reductive degradation. In the event of a severe pollution of an aquatic system with waste waters with a relatively high organic content then it may be expected that

the generated H_2S may have arisen from the proteolytic decomposition of these organics and not from the activities necessarily of the SRB.

Some basic information on the role of the sulfate reducing bacteria has been summarized in the University of Nebraska NebGuide[xlvi]:

> "(SRB) chemically change natural sulfates in water to hydrogen sulfide. (SRB) live in oxygen-deficient environments such as deep wells, plumbing systems, water softeners and water heaters."
>
> "Hydrogen sulfide gas occurs naturally in some groundwater. It is formed from decomposing underground deposits of organic matter such as decaying plant material."
>
> "Hydrogen sulfide gas produces an offensive "rotten egg" or "sulfur water" odor and taste in the water. In some cases the odor may be noticeable only when the water is initially turned on or when hot water is run. Heat forces the gas into the air which may cause the odor to be especially offensive in a shower."

Corrosion can be initiated by hydrogen sulfide generated from microbial activities. This forms a mechanism of electrolytic corrosion that is influenced by the microbial products of growth. This has led to the term "Microbiologically Influenced Corrosion" (MIC) being used to define these forms of events. Biocorrosion became recognized as not only stimulating general, pitting, crevice and stress embrittlement but is also capable of enhancing corrosion fatigue and hydrogen embrittlement and cracking[xlvii]. The links between corrosion and the SRB occurred over the period from 1965 to 1990 with a number of the important events listed in Table Six.

Table Six
Chronological Recognition of SRB as inducers of Biocorrosion

Date	Recognition	Reference
1968	Anaerobic corrosion and the SRB	xlviii
1971	Monograph on microbial corrosion	xlix
1971	Corrosion by SRB	l
1972	Mechanisms of sulfide corrosion by SRB	li
1984	SRB in localized corrosion of iron-based alloys	lii
1987	Role of microbial exopolymers in corrosion	liii
1992	Testing for microbiologically influenced corrosion	liv

The SRB are now widely recognized as being principal agents in the cause of MIC. In the water industry, the concerns relating to the SRB are three fold: (1) "rotten" egg odor; (2) corrosion and (3) unpleasant slimes and plugging[lv]. Contemporary concepts place the sulfate reducing bacteria as strictly anaerobic and not tolerant to the presence of oxygen[lvi] and would commonly be found

deeper down in the biofilms utilizing short chained fatty acids[lvii]. Essentially the SRB appear to be a distinctive part of complex bacterial consortia that tend, because of their inability to survive the presence of oxygen, deeper down in the consortial mass commonly formed by one or a multiplicity of biofilms. This was addressed in 1986 by Costerton, now Director of the Institute for Biofilm Engineering when he made the statements[lviii]:

> "In corrosion, a major event is that important organisms like the SRB's with their allies develop one set of physico-chemical conditions at point A while different physico-chemical conditions are established at point B by another consortium. The differences are sufficient to create a microbially induced chemical corrosion cell. In the last two years (1984-1986) much of the *magic* about corrosion has gone out granted that the current work has actually pointed to the corrosion potential existing between A and B as a result of these inter-consortial interactions."
> "In support of this phenomenon, 10^6 SRB's may be observed by fluorescent antibodies scattered evenly over a surface with no noticeable corrosion. However, where there are groupings, these can be matched up to corrosive micro pits (by scanning electron microscopy). Pigging presumably operates by blowing these grouped consortia apart at regular intervals and thus reducing the corrosion potential."

D.C. White, one of the major microbiologists who developed the phospholipid biomarkers for bacterial consortia, considered at the same 1986 meeting that[lix]

> "Particularly interesting is the facilitation of corrosion by bacteria and the latest work has been developed to show engineers (who don't believe that you can have anaerobic activity in aerobic systems!) that you can obtain corrosion if you allow bacteria to create micro-niches by metabolism using oxygen. Sulfate reducers and marine *Vibrio* species in seawater may attach to stainless steel and increase corrosion more than either of them would do separately under aerobic conditions."
> "It doesn't make any difference if you isolate sulfate reducing bacteria on a corroded piece of steel, they are always there; that's not what they want to attack. Sulfate reducing bacteria have got to have some help, it has to be made anaerobic and they don't *eat* many things. So if you go after the organisms that provide the SRB's with their food in the environment, it may be a lot easier to inhibit them"

One challenge in detecting SRB using the SRB-BART is therefore to be able to obtain a water sample that is likely to contain SRB given that:

1. SRB tend to inhabit the deeper reaches of the biofilms away commonly from exposure to oxygen and protected by other organisms that are able to utilize all of the oxygen in the water and generate suitable fatty acids to support the SRB.
2. Water sampled is likely to contain planktonic organisms and microbes inhabiting floating biocolloidal particles. The likelihood of the SRB being present is limited to only those biocolloids that contain anoxic (oxygen deficient) zones.
3. To obtain a water sample that is likely to contain high populations of SRB then the water would have to be anoxic and the biofilms interfacing

with the water should be disrupted to dislodge and suspend some of the SRB community that can then be detected in the SRB-BART.

The isolation through enrichment culture of *Desulfovibrio* (as one of the principal genera in the group I sulfate reducers: non-acetate oxidizers) is relatively easy on an anoxic lactate-sulfate medium to which ferrous iron is added[lx]. A reducing agent such as thioglycolate or ascorbate is also added to achieve a lower E_o'. When sulfate reducing bacteria grow, the sulfide formed from sulfate reduction combines with the ferrous iron to form black insoluble ferrous sulfide. This blackening not only indicates sulfate reduction, but the iron also ties and detoxifies the sulfide, making possible growth to higher cell yields. Purification can be achieved by subculturing into an agar roll tube or on a Petri dish in an anoxic glove box. For an alternative shake tube method, the original enrichment culture is added to a tube of molten agar growth medium, mixed thoroughly and sequentially diluted through a series of molten agar tubes. On solidification, individual cells distributed through the form black colonies that can now be removed aseptically, and the whole process repeated until pure cultures are achieved.

The American Petroleum Institute (API) recommended medium for the detection, differentiation, enumeration and titre determination of sulfate reducing bacteria in water[lxi]. The methods were recommended to be either aerobic or follow the procedures of Oberzill (1970)[lxii]. An API recommendation was that the incubation period should be at least four weeks at a temperature that did not differ from the original ambient water sample site temperature by more than $\pm 5^{o}C$.

The SRB-BART offers a number of distinctive advantages over the methodologies described above. These are listed below:

1. The environment suitable for the growth of the SRB is created using the original unmodified water sample so that there is a minimum of trauma to the incumbent SRB.
2. The consortia associated with the water sample are still able to flourish (and protect the SRB) but as the selective medium (Postgate's Medium C) diffuses up the water sample column then so there is a differential favoring of the SRB due to the high lactate concentrations diffusing upwards.
3. At the initial impact of the adding the water sample to the SRB-BART oxygenates the water to some extent but the SRB are protected by being intimately integrated into biocolloids or sheered organic particulate materials arising from the biofilms that may have been shattered during, or preceding, the sample event.
4. A reductive zone can rapidly be generated as a result of consortial respiratory activity. This most commonly will happen in the depths of the incubating SRB-BART where there is a very significant selective medium diffusing upwards from the base but it can also occur on the surfaces of

the floating ball where suspended biocolloids can become attached and create a cloistered reductive zone within which the SRB can flourish.

5. In incubating the water sample in such a dynamic environment with minimal stress applied to the SRB and the associated consortia means the net effect is that there is minimal impedance to the activities being generated by any SRB in the water sample. While the API recommends a four week incubation period for the standard recommended protocol described above, the SRB-BART is normally complete in ten days or less.
6. A clear differentiation of the SRB that was deep within tight biofilms and those that were protected by aerobic bacterial consortia growing in the more vulnerable layers of biofilms and in the biocolloidal particulates in the water. This differentiation would be the generation of a BB and a BT reaction respectively that could then extend to a BA in either case where there were very aggressive supportive consortia present.
7. A positive SRB-BART provides an enrichment culture from which pure cultures can be obtained by recognized laboratory procedures for the SRB.

2.4 Semi-Quantitative Evaluation

Semi-quantitative evaluation is achieved by an assessment of the aggressivity of the indigenous SRB 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 SRB 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 as the SRB-BART testers are able to; (2) the total inability of microscopic, spectrophotometric and laser particle counters to differentiate the SRB in the sample; and (3) the inability of the analytical techniques such as the adenosine triphosphate (ATP) to differentiate SRB. In using the SRB-BART tester for semi-quantitative evaluations there is an intrinsic problem resulting from the greater sensitivity of the SRB-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.
- Extinction dilution series of water samples to determine the impact of dilution on the length of the time lag using the SRB-BART tester
- Experiential evidence garnered by using the SRB-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 SRB-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 SRB-BART tester it was considered by the first distributor (Layne Inc., Kansas City, Ka[lxiii]) in 1988 – 89 that the BART testers would provide a simple, more robust but less sensitive field test to the dip-paddle. However the sensitivity of the SRB to oxygen limited to the ability to apply the dip-paddle for the detection of SRB. The dip-paddles for all of the targeted bacteria were discontinued and Mansuy (1999)[lxiv] 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 SRB-BART tester was more sensitive and convenient for the detection of target SRB in water.

2.4.2 Most Probable Number (MPN) for SRB

One of the standard techniques for the detection of SRB particularly in the oil and gas sector is the MPN method first established in 1960[lxv]. In this enumeration procedure, multiple serial dilutions are performed for the determination of the sulfate reducing bacteria. This can then allow the enumeration and determination of the sulfate reducing bacteria using the MPN method. This method remains today a standard most commonly used in analytical laboratories and in the field.

The test method involves a lactate-based medium differing from the SRB-BART in a number of ways (MPN changes to SRB-BART):

- Ferrous sulfate is replaced by ferrous ammonium sulfate
- K_2HPO_4 is replaced with KH_2PO_4
- Calcium sulfate replaces sodium sulfate
- Yeast extract is incorporated
- Sodium thiosulfate incorporated
- Nail excluded
- Thinner layer of mineral oil applied

The nail recommended is a 1" galvanized steel nail that has been washed in detergent, then methylene chloride, followed by 0.1N HCL before final rinsing in distilled water and drying either on a paper towel in a warm oven or using a hair dryer. This is followed by sterilizing for 15minutes by autoclaving.

The procedure involves three or five replicates of 10ml of the medium for each dilution to be tested. A single sterilized nail is added to each replicated vial. Dilutions use a standard 0.2% NaCl "buffer". One ml of the diluent forms the inoculum into

each replicate. The standard temperature is 30°C but "temperatures between 25°C and 30°C are acceptable". The tests are examined counted after 2 weeks as positive if there is a deposit of black iron sulfide either precipitated or on the nail. It is recommended that in the case of negatives the incubation continue for a third week. Revisions have been made to the basic procedure in 1989[lxvi] and 1998[lxvii]. The calculation of the MPN references WI MICR 005- Calculation of MPN[lxvii] . This method has formed the backbone of the standard method for the detection and enumeration of SRB and is widely used particularly in the oil and gas industry.

2.4.3 **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 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 with this product since the BART testers introduce 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 testers and would have led to a voiding of the patent. It was decided not to proceed but a contribution was made to the AWWA publication on Nuisance Bacteria[lxviii]. The 20th edition carried the Abd-el-Malek and Rizk (1960) as described above.

The SRB-BART tester in being presented for verification includes with other techniques and in many cases where the BART testers have been employed they have been selected as the only technique that would allow a convenient assessment of the bacteria in a water sample under field conditions.

2.5 Semi-Qualitative Evaluation

There has been less attention paid to the examination of general bacterial community structures as such but more to the ability to identify potential pathogenic or defined 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 has centered on finding the very specific rather than the more generalized approach of examining for the larger and more diverse communities. The SRB-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 SRB-BART tester.

2.5.1 Selection of SRB 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 SRB. In 1993, The Handbook of Microbiological Media[lxix] included a range of six of Postgate's media for the sulfate reducing bacteria. In the development of the SRB-BART tester the initial evaluation was of all six media in various combinations using natural samples from biofouled sites that had either, corrosion, black slime or taste and odor problems. From these studies, Medium C appeared to offer the broadest spectrum even though it used lactate as the principal source of fatty acid which would restrict determination to the group I sulfate reducers: non-acetate oxidizers[lxx]. Modifications were made to the medium:

Sodium sulfate replaced by calcium sulfate

Sodium thiosulfate added to neutralize any residual chlorine in the water sample

The reasoning behind these changes were based on the early development of the SRB-BART in which it was found that the sensitivity of the tester to SRB was severely reduced if there was residual chlorine in the water samples. This residual chlorine could lead to extended time lags or failure to detect the SRB at all. Neutralization of the chlorine with sodium thiosulfate gave good precision and shorter more consistent time lags. There was some concern over the use of sodium sulfate in the medium since this may act as a suppressant to any sodium-sensitive SRB. It was found that replacing the sodium with calcium sulfate gave a broader spectrum of positive detections.

Attempts were also made to develop the SRB-BART so that it could also detect the group II sulfate reducers: acetate oxidizers by the addition of acetate to the medium. However, the addition of acetate to the medium was found over the time period from 1989 to 1994 not to improve the sensitivity of the SRB-BART in the detection of SRB and so was discontinued.

Mineral oil was a further addition to the SRB-BART as a means of reducing the rate of oxygen entry into the liquid sample by floating a film of oxygen-impermeable oil on the surface. Through experimentation between 1991 and 1997, the volume of sterile mineral oil was gradually reduced to 0.05ml without any loss in the improved detection of SRB (through faster time lags and fewer false negatives).

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

Table Seven
Locations of SRB activity within the inner test vial of the SRB-BART when the sulfate reducing bacteria are present.

Position in SRB-BART	First reaction	Second reaction	Final reaction
Above ball			Coatings*
Around ball equator			
On underside of ball	Black particles[1] form	Blackening coats the surfaces[1]	
Mid-point of column	Clouds / turbidity**		Blackening of the walls[2]
Lower part of column			Blackening of the walls[2]
Conical base	Generation of a circle of blackening[3]	Completely black[3]	Blackening rises 2 to 5mm up the vertical walls

A BT reaction is noted as [1], reactions associated with the BA reaction are noted as [2] and reactions used to recognize a BB reaction are shown as [3]. *Coatings over the ball are rare and will occur much later and have no diagnostic value for the SRB-BART. **Clouds can often form in an incubating SRB-BART and this has been linked to the growth of anaerobic bacteria. Until 1997 this reaction was recognized as indicating the presence of anaerobic bacteria but was negative for the detection of SRB[lxxii]. This caused confusion for the users of the SRB-BART since the presence of clouding was not linked to the detection of SRB. This reaction was therefore removed from the diagnostic protocols. There are therefore two major primary focal sites for SRB detection (i.e., BB and BT) and one secondary reaction (BA).

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 various BART products and also the operation of the design, research and experimental development within the company. The various documents directly related to the BART testers are attached in appendix one and a summary of the documents are addressed 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 SRB-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 Eight:

Table Eight
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 SRB-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 SRB-BART testers is addressed in QI 30 (revision 1, 2 pages) and balling the SRB-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 SRB-BART tester product.

2.6.2 General Quality Management Relevant to the SRB-BART testers

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

Table Nine
ISO 9001 Documents Relating to the Production of SRB-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 117	SRB Medium Recipe	0	1
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
QF 117	SRB Batch Log Sheet	0	1
QF 147	SRB BART certificate of analysis	1	1

Additional forms and procedures are also more indirectly related to the production of the SRB-BART testers and these would be made available on request to Vincent Ostryzniuk, ISO Manager, DBI 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 SRB-BART testers (9 full SRB-BART testers with outer vials or 15 inner SRB-BART testers for use in the laboratory).

3 SRB-BART Verification Process

There are two stages in the verification of the SRB-BART testers. First, the SRB-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 SRB and associated consortia in the water sample being tested with the multiplicity of environments that are forming and changing within the water column of the tester.

In dealing with the SRB-BART testers two levels of evaluation are employed:

- Verification level assures that the SRB-BART tester meets with the criteria established to allow the tester to perform as claimed.
- Validation refers to the determination of the ability of the SRB-BART tester to determine the presence of the targeted bacteria as compared to standard methods.

In the claims for the SRB-BART tester verification refers to the ability of the specific instrument to detect the targeted SRB at the semi-quantitative and semi-qualitative levels.

3.1 **SRB-BART, Summary**

SRB is the shortened form of "Sulfate Reducing Bacteria" and embraces those bacteria that are able to reduce sulfate to hydrogen sulfide. This H_2S may then react with various forms of iron to generate black iron sulfides, generate electrolytic forms of corrosion in various metals and create nuisance odors and unpleasant slime-like masses. Presently the SRB are differentiated[lxxiii] into three major groups: (group I) non-acetate oxidizers; (group II) acetate oxidizers; and (group III) sulfur reducers. The SRB-BART mainly targets group I because it has been found in practice that most positive detections of SRB using the testers can be achieved using lactate as the primary fatty acid. Attempts were made to also include group III, the sulfur reducers, but it was found that the addition of sulfur tended to be inhibitory to many of the group I SRB.

SRB are obligate anaerobes and, as such, are sensitive to free oxygen which can be lethal. However the SRB can be protected from the presence of oxygen in water through co-habiting in bacterial consortia that generate polymeric matrices saturated with bound water to form protective slime coatings through which the oxygen cannot easily penetrate. In the development of the SRB-BART it has become clear that some SRB are able to co-habit within biofilms that are effectively surrounded by oxygen rich waters. When these SRB are present in the water sample then a BT reaction commonly occurs with blackening around the ball. On occasions when the SRB are growing in dense growths away from oxygen challenges then the form of growth in the SRB-BART tends to be BB.

3.1.1 Verification

The SRB-BART is manufactured using the recipe (QI 117, revision 0, 1 page) and the effectiveness of the SRB-BART is assessed using the SRB batch log sheet (QF 117, revision 0, 1 page) before a certificate of analysis is issued (QF 147, revision 1, 1 page). The finished SRB-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.
The primary claim is the differential selective activity of the SRB in the SRB-BART tester using modified Postgate's medium C. This medium has been recognized as being differential for sulfate reducing bacteria..

3.1.2 Verification of Claims, semi-quantitative

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

Table Ten
Relationship of Time Lag to Aggressivity and Population of SRB-BART

Time lag (days)	Aggressivity level	Population range cfu IRB/ml
0.05 – 6.0	High	1,000 to >1,000,000
6.05 – 8.0	Medium	999 to 11
8.05 – 10	Low	10 or less

Where there is an SRB reaction observed after ten days it is considered to be background and not aggressive. SRB activity/reaction has been observed after time lags as long as 42 days and moderately commonly with time lags of up to 14 days..

3.1.3 Verification of Claims, semi-qualitative

- The RPS emerging from the SRB-BART when activity is detected is relatively simple because there are only three recognized reaction codes that can be observed and five possible combinations (BB, BT, BA, BB-BA and BT-BA) and are described in Table Eleven below.

3.1.4 Validation of the SRB-BART tester

The SRB-BART tester employs a selective medium that creates environments that are changing quickly as a result of the formation of a redox front and diffusion of the selective modified Postgate's medium C 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 SRB 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 the group I SRB.

Table Eleven
Major RPS Groups Observed in the SRB-BART tester application to Waters

RPS	Family Type	Interpretation
BT	I	SRB associated with aerobic bacterial consortia in floating biocolloids or in sheered upper layers of biofilms
BT - BA	II	SRB associated with aerobic bacterial consortia in floating biocolloids and/or in sheered biofilms including some SRB from the deeper dense gels
BB	III	SRB cloistered deep in reductive zones forming tight dense biofilms with no presence of aerobic bacterial consortia
BB - BA	IV	SRB cloistered deep in reductive zones forming tight dense biofilms but some aerobic bacterial consortia are present
BA	V	Very aggressive SRB population including aerobic bacterial consortia and SRB from deep dense biofilms in the reductive zones.

3.2 Primary validation of the SRB-BART

The SRB-BART has been employed in a large number of investigations involving a mixture of approximately 105,000 in retail sales and 40,000 in various research projects. Demand for the SRB-BART testers has been driven by the following factors:

- Test is complete in ten days rather than three weeks
- Two distinctive initial reactions (i.e., BT and BB) can be easily observed and the time lag assigned
- Only one final reaction occurs (BA) which is clearly evident by extensive blackening
- No dilutions are required in the operation of the tester
- No set-time needed to prepare the tester prior to adding the sample
- Interpretation is easy with five possible combinations of reactions
- Replicable results achievable
- Room temperature used for incubation except under unusual circumstances

Major publications on the SRB-BART have been released (Table Twelve). The historical development of the SRB-BART is included in Table Thirteen and illustrates some of the principal users who have reported their findings on the SRB-BART in a documented manner. Many more users have reported using the SRB-

BART with very confident and replicable results but these are not documented and so are not included in this document.

Table Twelve
Major Publications Relating to the Use of the SRB-BART testers

Year	Topic	Reference
1993	Use of BART testers in ground water	lxxv
1998	The use of BART testers in wells rehabilitation	lxxvi
1999	The use of BART testers in control of biofouling	lxxvii
2000	Use of BART testers in determining well biofouling	lxxviii
2000	Identification of bacterial consortia using BART testers	lxxix

Table Thirteen
Historical Development and Verification of the SRB-BART

1986	Concept developed by Roy Cullimore and George Alford during the AWRC symposium on biofouled aquifers	
1987	Research concentrated on the SRB-BART. First use of the BART in well rehabilitation at the Grenada Dam, Mississippi by Mircon Consulting (Estevan) Ltd	lxxx
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	lxxxi
1988 - 2001	BART testers (IRB and SRB) were used to detect biofouling and trigger rehabilitation treatments in Waverley, Tennessee – still being used to present time for preventative maintenance	lxxxii
1988 - 1990	BARTs used in the development of certification processes for Organic Farmers	lxxxiii
1989	SRB-BART described in the Canadian Water Well Journal	lxxxiv
1989	Frequency of SRB in Canadian ground waters discussed	lxxxv
1989	Layne –Western Company, Inc releases first description of the use of BARTs in well rehabilitation	lxxxvi
1990	General description of the BAT (BART) testers including the SRB-	lxxxvii
1990	SRB-BART discussed at an International Conference on Microbiology in Civil Engineering	lxxxviii
1990	U.S. Patent issued on the BART tester	lxxxix
1990	Comparison of the use of the BART technology with the standard methods	xc
1990	The use of the SRB-BART testers in the determination of the treatment effectiveness of the rehabilitation of water well biofouling	xci

1990	The use of the BART testers to determine the effectiveness of the treatment of biofouled water wells	xcii
1990	SRB-BART tester system described by Layne-Western Company Inc	xciii
1990	BARTs are used by Ortech International, Missisauga, Ont to determine the effectiveness of in situ barriers to control BTEX	xciv
1990	BARTs used in the evaluation of Biofouling of water wells in Newcastle, N.B	xcv
1992	SRB-BART testers were used on the examination of rusticles recovered from the RMS Titanic (IMAX 1991 expedition)	xcvi
1992	SRB-BART included in the generation of the well plugging risk index	xcvii
1993	First full description of the SRB-BART with protocol and interpretation methodologies	xcviii
1993	Discussion of the use of the SRB-BART in an AWWA study of the evaluation and restoration of water supply wells	xcix
1993	Atomic Energy of Canada uses the SRB-BART for field analysis	c
1994	First full SRB-BART interpretation and Reaction Chart as a part of the full set of BART tester	ci
1994	SRB-BART used as a part of the BART strategy to determine biofouling of DCE in leachate	cii
1996	BART tester recognized in a major book “Microbial Quality of Water Supply in Distribution Systems” by Edwin Geldreich	ciii
1996	Japanese version of the BART comparator chart prepared	civ
1997	Description of the use of the BART testers on the RMS Titanic in the 1996 Discovery Channel expedition	cv
1997	Hach Corporation includes the use of the BART testers on the RMS Titanic in 1996	cvi
1997	Leggette, Brashears & Graham Inc (LBGI)of CT announce agreement with the AWWA research foundation to determine suitable methods for well rehabilitation, the BART testers are included as the only suitable field test system to meet the needs	cvii
1997	SRB-BART used by Canada Agriculture and Agri-Food to determine fouling problems in water wells in the Kneehill district of Alberta	cviii
1997	Public advisory was released in Kneehill M.D., Alberta concerning biofouling including SRB by PFRA	cix
1998	SRB-BART used in a joint project with PFRA to assess effectiveness of UAB treatment	cx
1998	LBGI first periodic report SRB-BART protocol and sampling procedures	cxi

1999	LBGI field trials using the SRB-BART at Birmingham, CT and Mosinee, WI	[cxii]
1999	BART methods including SRB- discussed in "Iron and Manganese Removal Handbook published by the American Water Works Association	[cxiii]
1999	Use of the SRB-BART discussed in Water Well Rehabilitation	[cxiv]
1999 - 2000	Champion Technologies Ltd undertake evaluation of the SRB-BART against standard and elect to use the SRB-BART as their corrosion marker at all field sites	See note 1
1999 - 2001	National Ground Water Association organizes a series of two-day workshops on water well rehabilitation including the use of the SRB-BART in Denver, 1999; Milwaukee, 2000, Las Vegas, 2001.	[cxv]
2000	Discussion of the BART test platform placed on the RMS Titanic in 1998 that included the SRB-BART	[cxvi]
2000	Expanded description of the SRB-BART and interpretation methodologies	[cxvii]
2000	Determination of microbial composition of rusticles using the BART testers	[cxviii]
2000	LBGI AWWARF study at Mosinee, WI, Ames, IOWA; Sarasota, FL; Houston, TX; North Battleford, SK, Canada.	[cxix]
2000	PFRA releases final phase report including the use of the SRB-BART	[cxx]
2000	U.S. Army Corp of Engineers release management engineering pamphlet on the rehabilitation of injection and extraction wells involving the SRB-BART	[cxxi]
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 SRB-BART	[cxxii]
2001	Comprehensive evaluation of the BARTs to predict biofouling in porous media as a joint PFRA / DBI project	[cxxiii]
2002	AWWA is going to publish in Opflow the use of the BARTs as a part of an article by Larry Thomas	[cxxiv]

Note 1: Mr. T. Elwadia of Champion Technologies Ltd. Calgary was frustrated with the difficulty of getting his field staff in the oil fields to conduct the standard SRB tests using sequential dilutions. A comparison was undertaken late in 1999 and it was found that for 16 sixteen samples:

- SRB-BART had one false negative (6%) while the 10K saline had 38% and the 25K saline had 13%
- SRB-BART functioned at the one ml sensitivity level while the standard functioned at the 100ml level
- SRB-BART was easy and convenient to set up and read compared to the standard method since no dilutions were involved

- SRB-BART mimics natural at-site conditions more accurately than the standard method
- SRB-BART test completed in less than ten days rather than twenty one days using the standard method
- Technicians found the reading of the SRB-BART to be convenient.
- Champion decided that because of the superior ease-of-use, greater sensitivity and faster turn-around of data to switch over to the SRB-BART for all of their field work mainly related to corrosion-prevention in the oil fields known to be caused by SRB.

4 Summary of Claims for Verification of the SRB-BART

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

4.1 Definition of SRB

SRB for the purposes of the test shall be primarily classified as the group I, non-acetate oxidizers that would include the most ubiquitous genus *Desulfovibrio*. The nature of the SRB-BART employing a vertical array of different and changing lateral environmental niches would allow the undiluted indigenous organisms to degrade the lactate in the tester to acetate. In this event then there is a potential for the group II sulfate reducers: acetate oxidizers to also be detectable. The definition of SRB that could be detected using the SRB-BART is therefore limited to the fatty acid oxidizers. The Dissimilatory sulfur bacteria would not be detected unless sulfur became available through the oxidation of H_2S to sulfur by *Beggiatoa* or deposition of sulfur by the green or purple sulfur bacteria. This generation of sulfur would likely be a tertiary event that would not occur in the recommended incubation time of ten days. SRB as a term is therefore essentially limiting the test to the detection of sulfate reducing bacteria and not sulfur reducers.

4.2 Selective Culture Medium for the SRB

Postgate's medium C has been well recognized as a standard medium for the enrichment of the SRB (group I). In the early development of the SRB-BART it was found through practical experiential observations that the medium could be improved in it's selectivity by the replacement of sodium sulfate with calcium sulfate (to reduce the impact of sodium on salt-sensitive SRB), and the addition of sodium thiosulfate to neutralize any residual chlorine that may be in the water sample, and the addition of mineral oil to reduce the downward diffusion of oxygen around the floating ball. The form of the modified Postgate's C medium is as a crystallized pellet implanted from x10 strength concentrates. The iron in the medium as ferrous sulfate generates the iron sulfide for positive detection either in the base (BB) around the ball (BT) or on the walls (BA) without the need to introduce a sterilized galvanized nail (used in the standard methods).

4.3 Sample Management in the SRB-BART test

The water sample in the SRB-BART test is used directly without dilution. This means that the indigenous microflora is not impacted by the impacts of dilution and that the technician conducting the test can apply 15ml of sample directly to the SRB-BART. This compares favorably against the standard method that involves a diluent that has to be adjusted to the salt concentration in the water sample (e.g. distilled water, phosphate buffer, 10K or 25K saline).

4.4 The Novel Format Created in the SRB-BART

A major feature of the patent is that an aspect ratio is created that causes the indigenous microflora 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 is the SRB-BART test is consumed by the indigenous microflora.

4.5 Incubation of the SRB-BART

Through practice, it has been found that room temperature with an average of $22^{o}C$ 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 SRB-BART it should be kept in a location where the temperature is not likely to radically fluctuate and away from direct sunlight. The SRB-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 SRB-BART tester

The recommended time frame for the incubation of the SRB-BART is ten days. Under some circumstances positive detections may continue until the 14^{th} day but these would be considered as "background". The standard test employs a 21 day standard with readings possible after 14 days. The SRB-BART is therefore faster and will commonly generate significant positives in less than 8 days.

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

SRB activity is recognized by the formation of black iron sulfides that can easily be recognized as it forms in the conical base (as a BB), on the surface of the floating ball (as a BT) or over the walls (as a BA). All three activities are easy to recognize and the incubation time to the first observation of the activity should be recorded. Since there are no dilution series, the observation is of the original water sample in the SRB-BART tester and so there is less technician time employed in recording a positive than would be the case for the standard test.

4.8 Semi-Quantitative Evaluation, Aggressivity

Aggressivity of the SRB 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 6 days or less. Medium aggressivity would have a time

lag of between 6 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 SRB would be "background" and therefore not significant.

4.9 Semi-Qualitative Evaluation, SRB consortial identification

Because there are three distinct activities that can be observed in the SRB-BART tester, it is possible to undertake a semi-qualitative identification of the bacterial consortia associated with the SRB. This is described in Table Eleven with five possible consortial family combinations. This evaluation is not possible using the standard test.

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

The confirmatory method to confirm the presence of SRB was first developed in 1989 at the Ontario water laboratories. Here, a series of thirty samples were run on both the SRB-BART and the standard "nail" method. Eighteen went positive for the SRB-BART and only seven for the standard method. When the positive SRB-BART tests were sub-cultured into the standard "nail" method, all went positive showing that the SRB had grown in the SRB-BART but not in the standard method until enriched by incubation in the SRB-BART. This indicated that the standard "nail" method had generated 60% false negatives compared to the SRB-BART. This protocol was also used by Champion Technologies Ltd in 1999 with similar results. Note the Ontario protocol is not specifically included in the body of the document because the scientist who described the experiment did not follow up with documented discussion of these findings.

5 Primary Claim

The SRB-BART generates, when charged with a water sample, a sufficient diversity of environments that will encourage the determination of observable activities of the SRB within the water sample being tested. From experiences to-date the SRB-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 SRB-BART tester are sufficient for the verification of the Biodetector as a suitable system for the detection of SRB in water-based samples. These would be subject to the following limitations:

1. The limits of detection for the SRB in a given water sample would be 67cells/l.
2. Any water sample taken for testing using the SRB-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 SRB-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 SRB-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.

6 References

[i] Beijerinck, W.M. (1895) Über *Spirillum desulfuricans* als Ursache Sulfatreduktion. Centrlblatt für Bakteriologie und Parasitenkunde, Abt 2 **1**:115 - 117

[ii] van Delden, A (1903) Beitrag zur Kenntnis der Sulfatreduktion durch Bacterien. Centralblatt for Bacteriologie und Parasitenkunde, Abt 2 11:81 – 94 113 - 199

[iii] Baars, E.K. (1930) Over Sulfaatreductie door Bacterien. Doctoral thesis. University of Delft. Delft, Holland. W.D. Meina N.V.

[iv] Kluyver, A.J. and C.B. van Neil (1936) Prospects for the natural system of classification of bacteria. Zentrallblat für Bakteriologie, Paraistenkunde, Infectionsfrankheiten, und Hygiene, Abt. 2 94:369 - 403

[v] Postgate, J. R. (1959) Sulfate reduction by bacteria. Ann. Reviews of Microbiology **13**:505 - 520

[vi] Postgate, J.R. (1965) Recent Advances in the study of sulfate-reducing bacteria, Bacteriological Reviews **29**:425 - 441

[vii] Postgate, J.R. and L.L. Campbell (1966) Classification of *Desulfovibrio* species, the non-sporulating sulfate reducing bacteria Bacteriological Reviews **30**:732 - 738

[viii] Pfennig, N, Widdell, F and H.G. Trüper (1981) the Dissimilatory Sulfate-Reducing Bacteria in the Prokaryotes, Volume 1, Springer Verlag, NY. Chapter 74 926 – 940.

[ix] Madigan, M.T., Martinenko, J.M. and J. Parker (1997) Brock Biology of Microorganisms, Prentice Hall, NJ. 671 – 676.

[x] Tiller, A.K. (1990) Biocorrosion in Civil Engineering in Microbiology in Civil Engineering (ed. P Howsam) E. & F.N. Spon London 24 - 38

[xi] American Water Works Association (1995) Sulfur Bacteria Chapter 3 in AWWA Manual M7 , AWWA Denver, Co 19 - 26

[xii] Cullimore, D.R. (2000) Microbiology of Well Biofouling Lewis Publishers / CRC Press. Boca Raton, FL pages 432: 52 – 56.

[xiii] Cullimore, D. Roy (2000) Practical Atlas for Bacterial Identification pages 209, figures 10.41 and 10.42.

[xiv] Franson, M.A.H (1986) Identification of Iron and Sulfur Bacteria, American Public Health association Washington, DC 1019 - 1030

[xv] American Water Works Association (1995) Manual of Water Supply Practices, Problem Organisms in Water: Identification and Treatment, Chapter Two Iron Bacteria pages 153:7 - 17

[xvi] Postgate, J.R., Odom, J.M. and R. Singleton (1992) The Sulfate Reducing Bacteria: Contemporary Perspectives published by Springer-Verlag as a part of the Brock series in Contemporary Bioscience pages 289

[xvii] Deveraux, R and D. Stahl (1992) Phylogeny of Sulfate-Reducing Bacteria in Postgate, J.R., Odom, J.M. and R. Singleton (1992) The Sulfate Reducing Bacteria: Contemporary Perspectives published by Springer-Verlag as a part of the Brock series in Contemporary Bioscience pages 289:131 - 160

[xviii] Devereux, R., Hines, M.E. and D.A. Stahl (1996) S Cycling: Characterization of Natural Communities of Sulfate-Reducing Bacteria by 16S rRNA Sequence Comparisons Microb. Ecol 32:283-292
[xix] K. Bade (2000) Survival of sulfate-reducing bacteria in oxic oligotrophic environments related to drinking water, Ph.D. thesis Technical University of Berlin 2124 Kb (.pdf)
[xx] www.biochem.ucl.ac.uk/bsm/proL.ig/pdbEntries/2cy3/
[xxi] Caldwell, D.E. (1986) Microbial Colonization of Surfaces in Proceedings of the AWRA International Symposium on Biofouled Aquifers: Prevention and Restoration (ed. D. R. Cullimore) published by AWRA, Bethesda, Maryland p 7 – 11.
[xxii] Characklis, W.G., Cunningham, A.B., Escher, A and D. Crawford (1986) Biofilms in Porous Media in Proceedings of the AWRA International Symposium on Biofouled Aquifers: Prevention and Restoration (ed. D. R. Cullimore) published by AWRA, Bethesda, Maryland p 57 – 79.
[xxiii] Wojcik, W. and M. Wojcik, M. (1986) Monitoring of Biofouling in Proceedings of the AWRA International Symposium on Biofouled Aquifers: Prevention and Restoration (ed. D. R. Cullimore) published by AWRA, Bethesda, Maryland p 109 – 121.
[xxiv] Cullimore, D.R. (editor) 1986 IPSCO Think Tank on Biofilms and Biofouling in Wells and Groundwater systems, published by the Regina Water Research Institute, University of Regina, Canada 86 pages
[xxv] Vinebrooke, R. and D. R. Cullimore (1998) Natural Organic Matter and the Bound-Water Concept in Aquatic Ecosystems published in the International Reviews of Environmental Contamination and Toxicology, Springer Verlag 155: 111-127
[xxvi] Cullimore, D.R. (2000) Microbiology of Well Biofouling Lewis Publishers / CRC Press, Boca Raton FL pages 435: 388 – 389, figure 67 & 68
[xxvii] Lebeden, J., Jacques, D. and H. Rohde (2000) Rural Municipality of Mount Hope #279, Water Well Inventory and Microbiological Activity Assessment SWWI document, Agriculture and Agri-Food Canada, Ottawa pages 22
[xxviii] Cullimore, D.R. (2001) The Development and Validation of Microbiological Methods for the Bioprospecting for Oil and Gas Reserves using Ground Water Sources Report to Petroleum technology Research Center, Regina Research Park, Regina SK pages 13.
[xxix] Cullimore, D.R. (1993) Practical Manual of Groundwater Microbiology, Lewis Publishers / CRC press Figure 45, page 308 and text relating to reaction X p 309
[xxx] Mansuy, N, Nuzman, C and D.R.Cullimore (1990) Well Problem Identification and its Importance in Well Rehabilitation in Water Wells: Monitoring, Maintenance, rehabilitation (edited by P. Howsam) E&F Spon, London 87 - 99
[xxxi] Gehrels, J and G.Alford (1990) Application of Physicochemical Treatment Techniques to a Severely Biofouled Community Well in Ontario, Canada Rehabilitation in Water Wells: Monitoring, Maintenance, rehabilitation (edited by P. Howsam) E&F Spon, London. 219- 235
[xxxii] Cullimore, D.R. (1990) An Evaluation of the Risk of Microbial Clogging and Corrosion in Boreholes Rehabilitation in Water Wells: Monitoring, Maintenance, rehabilitation (edited by P. Howsam) E&F Spon, London. 25 -34
[xxxiii] Lindenbach, S.K. and D.R.Cullimore (1989) Preliminary in vitro Observations on the bacteriology of the black plug layer phenomenon associated with the biofouling of golf greens. J. Appl. Bact 67: 11 - 17
[xxxiv] Cullimore, D.R. (1993) Practical Manual of Groundwater Microbiology published by Lewis Publishing, Michigan ISBN 0-87371-295-1. 412 pages
[xxxv] Smith, S.A. (1995) Monitoring and Remediation Wells: Problem Prevention, Maintenance, and Rehabilitation Lewis Publishing / CRC Press Boca Raton FL pages 184: 83 – 92.
[xxxvi] Mansuy, N (1999) Water Well Rehabilitation Lewis Publishing / CRC Press Boca Raton FL pages 174: 102 - 106
[xxxvii] Cullimore, D.R. (2000) Microbiology of Well Biofouling published by Lewis Publishing / CRC Press, Boca Raton, Florida ISBN 1-56670-400-6 435 pages.
[xxxviii] Cullimore, D.R. (2001) Practical Atlas for Bacterial Identification published by CRC Press, Boca Raton, Florida. ISBN 1-56670-392-1 209 pages
[xxxix] Atlas, R.M. and L.C. Parks (1993) Handbook of Microbiological Media CRC Press Boca Raton FL pages 1077: 557
[xl] Atlas, R.M. and R. Bartha (1981) Microbial Ecology: Fundamentals and Applications Addison-Wesley Publishing Co. Reading, Mass, pages 560 Figure 5.32 & 159 - 161

[xli] Tortora, G.J., Funke, B.R. and C.L.Case (2001) Microbiology, An Introduction published by Addison Wesley Longman, NY, ISBN 0-8053-7543-0. p 157 - 158
[xlii] Hattori, T. (1988) The Viable Count: Quantitative and Environmental Aspects. Published by Science Tech Publishers, Madison, Wisc. With Springer-Verlag, NY 88 pages.
[xliii] Pfennig, N., Widdel, F. and H.G. Trüper (1981) The Dissimilatory Sulfate-Reducing Bacteria in The Prokaryotes edited by M.P. Starr et al published by Springer-Verlag NY 1102 (volume one): 929-932
[xliv] World Health Organization (1981) Hydrogen Sulphide as Environmental Health Criteria 19 published by the World Health Organization, Geneva pages 48, 1.1.2 , 1.1.6, 3.1, and 7
[xlv] Cooper, R.C., Jenkins, D. and L.Y. Young (1976) Aquatic microbiology manual, Texas, University of Texas (also Figure one in reference xliv)
[xlvi] Varner, D., Skipton, S., Hay, D.L. and P. Jasa (2001) Drinking Water: Sulfates and Hydrogen Sulfide, Neb Guide G96-1275-A, published by Institute of Agriculture and Natural Resources, University of Nebraska, pages 7
[xlvii] Edyvean, R.G.J., Thomas, C.J., and I.M. Austen (1985) Biologically Induced Corrosion **NACE 8** (ed. S.C. Dexter) National Association of Corrosion Engineers, Houston, Texas.
[xlviii] Hamilton, W.A.(1968) Sulfate reducing bacteria and anaerobic corrosion. Ann. Rev. Microbiology39:195-217
[xlix] Booth, G.H. (1971) Microbial Corrosion. M&B Monograph CE/1 Mills and Boon, London
[l] King, R.A. and Miller, J.D.A (1971) Corrosion by sulfate reducing bacteria Nature 233:491
[li] Mara, D.D. and D.S.A. Williams (1972) The mechanism of sulfide corrosion by SRB in Biodeterioration of Materials, Volume 2 (ed. A.H. Walters and E.H. Hueck-van-der Plas) Applied Science, Barking, UK
[lii] Gragnolina, G. and O.H. Tuovinen (1984) The role of sulfate reducing and sulfur oxidizing bacteria in the localized corrosion of iron-base alloys: a review in International Biodeterioration 20:9-26
[liii] Ford, T.E., Maki, J.S. and R. Mitchell (1987) Involvement of microbial exopolymers in Biodeterioration of metals in Biodeterioration 7 (ed. Houghton D.R., Smith, R.N. and H.O.W. Eggins) Elsevier Applied Science.
[liv] Kearns, J.R. and B.J. Little (1994) Microbiologically Influenced Corrosion Testing Proceedings of an ASTM meeting, November, 1992, Miami FL ISBN 0-8031-1892-9 pages 297
[lv] Cullimore, D.R. (2000) Microbiology of Well Biofouling Lewis Publishers / CRC Press pages 435: 166-167
[lvi] Lueschow, L.A. and K.M. Mackenthun (1962) Detection and Enumeration of Iron Bacteria in Municipal Water Supplies J. Amer. Water Works Association 54:751-756
[lvii] Geldreich, E.E. Microbial Quality of Water Supply in Distribution Systems CRC Press Boca Raton FL pages 504: 235-236
[lviii] Costerton (1986) Conceiving, culturing and controlling biofilms in IPSCO Think Tank on Biofilms and Biofouling (ed. D.R. Cullimore) published by RWRI University of Regina ISBN 0-7731-0097-0 pages 119:3-5
[lix] White, D.C. (1986) Biochemistry of Biofilms in IPSCO Think Tank on Biofilms and Biofouling (ed. D.R. Cullimore) published by RWRI University of Regina ISBN 0-7731-0097-0 pages 119:13-15
[lx] Madigan, M.T., Martinko, J.M. and J. Parker (2000) Brock Biology of Microorganisms Prentice-Hall NJ,991: 498-502 Table 13.21
[lxi] American Petroleum Institute (1965) Recommended Practice for Biological Analysis of Subsurface Injection Waters Volume 38, 2nd edition
[lxii] Oberzill, W (1970) Quantitative Erfassung sulfatreduzierender Bacterien – Zbl. Bakt. II 124:91-96
[lxiii] Layne Western Co., (1988) promotional poster displaying the various BART products and agar dip paddles manufactured by Droycon Bioconcepts Inc. for distribution through Layne, poster sized full cover printed on both sides.
[lxiv] Mansuy, N (1999) Water Well Rehabilitation. Lewis Publishing 102 - 104
[lxv] Abde-el-Malek, Y and S.G. Rizk (1960) Culture of *Desulfovibrio desulfuricans* Nature 185:635-636
[lxvi] Methods of Microbiological and Toxicological Analysis of Waters and Wastewaters, and Sediments NWRI 1989 Sulfate Reducers, Desulfovibrio, 37 - 38
[lxvii] American Public Health Association (1998) Standard Methods for the Examination of Water and Wastewater 20th ed.
[lxviii] Cullimore (1995) Sulfur Bacteria in Nuisance Bacteria AWWA publication
[lxix] Atlas, R.M. and L,C. Parks Handbook of Microbiological Media CRC Press pages 1074: 457 - 458

[lxx] Madigan, M.T., Martinko, J.M. and J. Parker (2000)Brock Biology of Microorganisms Prentice-Hall NJ pages 991: 500 Table 13.21
[lxxi] Prescott, L.M., Harley, J.P. and D.A. Klein (1996) Microbiology published by Wm C Brown, Dubuque, 831– 832 Figure 41.1
[lxxii] Cullimore, D.R. (1993) Practical Manual of Ground Water Microbiology page 309
[lxxiii] Madigan, M.T., Martinko, J.M. and J. Parker (2000)Brock Biology of Microorganisms Prentice-Hall NJ pages 991: 500 Table 13.21
[lxxiv] Cullimore, D.R. (1993) Practical Manual of Ground Water Microbiology see correlations between time lag and population pages 309
[lxxv] Cullimore< D.R. (1993) Practical Manual of Groundwater Microbiology published by Lewis publishers, Boca Raton, 412 pages see pages 276 - 315
[lxxvi] Mansuy, N. Water Well Rehabilitation published by Lewis Publishers, Boca Raton, 174 pages see pages 102 – 111
[lxxvii] Alford, G and D.R. Cullimore (1999) The application of heat and chemicals in the control of biofouling events in wells published by Lewis publishers, Boca Raton, 181 pages see pages 51, 71,88, 93, 153
[lxxviii] Cullimore, R (2000) Microbiology of Well Biofouling published by CRC Press, Boca Raton 435 pages see pages 137 - 280
[lxxix] Cullimore, D.R. (2000) Practical Atlas for Bacterial Identification published by CRC Press, Boca Raton 209 pages see pages 177 - 195
[lxxx] Mansuy, N. (1987) Comparison of Well Rehabilitation Methods at Grenada dam, Mississippi report contracted to the U.S. Army Corp of Engineers 5 pages, 42 pages data
[lxxxi] Cullimore, D.R. (1990) Biofouling of Extraction Wells at Stone Container Paper & Pulp Mill, Missoula, Montana RWRI report, University of Regina also Cullimore, D.R. (2000) Microbiology of Well biofouling Figure 66.
[lxxxii] Cullimore, D.R. (2000) Microbiology of Well Biofouling BART Test (Waverley Scenario) page 94 - 96
[lxxxiii] Cullimore, D.R., Mushkin, M., Ostryzniuk, E and F. Alforo (1988) Biological Assay of Soils RWRI No. 58 University of Regina pages 15
[lxxxiv] Cullimore, D.R. (1989) Looking for Iron Bacteria in Water in Canadian Water Well 15(3) 10 - 12
[lxxxv] Mansuy, N and D.R. Cullimore Treating the Source not the Symptoms in Water in Canadian Water Well 15(3) 8 - 9
[lxxxvi] Layne-Western Company, Inc (1989) Well Rehabilitation Services: Testing for Bacteriological Activity, two page release, May, 1989
[lxxxvii] Cullimore, D.R., Alford, G. and N. Mansuy (1990) Studies on the Utilization of a Floating Intercedent Device (FID) in the Biological Activity Test (BATS) to Identify the presence and activity of Various Bacterial Groups in Groundwater contributed paper 3-2 First International Symposium on Microbiology of the Deep Subsurface U.S Department of Energy, Florida 55:2
[lxxxviii] Cullimore, D.R. (1990) Microbes in Civil Engineering Environments: Biofilms and Biofouling in FEMS Symposium No. 59 Microbiology in Civil Engineering (ed. P. Howsam) 15 – 23.
[lxxxix] Cullimore, D.R. and G. Alford Method and Apparatus for Producing Analytic Culture, U.S. Patent 4.906,1990
[xc] Cullimore, D.R. (1990) An evaluation of the risk of microbial clogging and corrosion in boreholes in Water Wells monitoring, maintenance, rehabilitation edited by P Howsam, E & F.N. Spon p25 - 34
[xci] Mansuy, N, Nuzman, C and D.R. Cullimore (1990) Well Problem Identification and it's importance to well rehabilitation in Water Wells monitoring, maintenance, rehabilitation edited by P Howsam, E & F.N. Spon p87 -99
[xcii] Gehrels, J and G. Alford (1990) Application of Physico-chemical Treatment Techniques to a severely biofouled community well in Ontario, Canada in Water Wells monitoring, maintenance, rehabilitation edited by P Howsam, E & F.N. Spon p219 - 235
[xciii] Layne-Western Company Inc (1990) Biological Activity Reaction Tests: BARTS, six page folded pamphlet LW-796 7/90.
[xciv] Cullimore, D.R., Abimbola, A., Rheil, J. and M. Mushkin (1990) I Situ Intercedent biological Barriers for the Containment and Remediation of Contaminated Groundwater RWRI report 1b University of Regina 143 pages

[xcv] Cullimore, D.R., Abimbola, A., Rheil, J and M. Mushkin (1990) Evaluation of the Potential Biofouling of Water Wells, Newcastle, N.B. RWRI Report No. 64a University of Regina, pages 175
[xcvi] Stoffyn-Egli, P and D. Butley (1992) The Titanic, 80 years later, Initial Observations on the microstructure and biogeochemistry of corrosion products poster presentation by the Atlanta Geoscience Centre, Halifax to the 1992 Geological Survey of Canada Conference. 1 poster.
[xcvii] Cullimore, D.R. and G. Alford (1992) Development of a computer assisted program to project the well plugging risk index (wpri) for existing or planned water wells in Ground Chemistry: Implications for Construction (ed. A. B. Hawkins) Proc. Of the International Conference of Ground Chemistry and Microbiology for the Construction Industry A.A. Blakema/Rotterdam/Brookfield/1997 5-14 : 591-605
[xcviii] Cullimore, D.R. (1993) Practical Manual of Ground water Microbiology, 295 - 302
[xcix] Borch, M.A., Smith, S and L. Noble Evaluation and restoration of Water Supply Wells published by the American water Works Association ISBN 0-89867-659-2 pages 99 - 101
[c] Stroes-Gasgoyne (1993) discussion in Nuclear Fuel Waste Management Review: Microbial Tutorial pusblished by Scientific Review Group, Federal Environmental Review Agency, Ottawa pages 134:14
[ci] DBI (1995) BART interpretation and reaction Chart 1 poster full color
[cii] Rowe, K (1994) A Laboratory Investigation into the Degradation of Dichloromethane, Geotechnical Research Center, University of Western Ontario pages 91:12, 60(Table 9)
[ciii] E. Geldreich (1996) Bart Test Kits in Microbial Quality of Water Supply in Distribution Systems published by Lewis Publishers ISBN 1-56670-194-5, pages 504: 236 – 237
[civ] DBI BART Reaction Comparator Chart Japanese version for Jetro, Japan. 1 poster double sided
[cv] Garzke, W.H., Brown, D.K., Mathias, Cullimore, D.R., Livingstone, D., Leighly, H.P., Foecke, T., and A. Sandiford (1997) Titanic, The Anatomy of a Disaster: a report of the marine forensic panel. Proceedings of the Annual Meeting, Technical Sessions, Society of Naval Architects and Marine Engineers, 1-1 to 1-47 (1-35 to 1-38)
[cvi] News and Notes Hach 11(2) Lit. No. 7448 and Microscoop News Lit No. 7619 and MicroTest News Lit No.4256
[cvii] Leggette, Brashear & Graham Inc (1997) well rehabilitation project funding agreement with American water Works Association Research Foundation, project 464 pages 68: 001 (BART recognition) 004 introduces the BART testers. 016 defines the use of the BART kits in outline
[cviii] PFRA (1997) Ground water and Water Wells in the Municipal District of Kneehill, Alberta prepared by PFRA Earth Science Division. May 1997 41 pages summary Figure 16
[cix] PFRA (1997) Sustainable Water Well Initiative, Biofouling and Water Wells in the M.D. of Kneehill, Alberta, June 1997 four page folded brochure.
[cx] PFRA / DBI (1998) City of North Battleford Well 15, 1997 Field Test of UAB 26 pages 4 appendices
[cxi] Leggette, Brashear & Graham Inc (1997) well rehabilitation project funding agreement with American water Works Association Research Foundation, project 464 first periodic report appendix III: 20-22 & 47-53
[cxii] Leggette, Brashear & Graham Inc (1997) well rehabilitation project funding agreement with American water Works Association Research Foundation, project 464 fourth periodic report appendix II Birmingham CT 3 – 10 & table 4; Appendix III: 1 - 5
[cxiii] Sommerfeld, E.O. (1999) Iron and Manganese Handbook published by the American Water Works Association, Denver pages 158: 45
[cxiv] Mansuy, N (1999) Water Well Rehabilitation published by Lewis Publishers / CRC Press, Boca Raton pages 174: 103 - 108
[cxv] National Ground Water Association, Westerville, Ohio, workshops on prevention, rehabilitation and maintenance for wells, two days with three speakers including a one-hour session on the BART testers including the SRB-BART
[cxvi] Cullimore, R and L. Johnston (2000) Science and the RMS Titanic: A Biological Odyssey Voyage 32: 172-176
[cxvii] Cullimore, D.R. (2000) Chapter 5, About the BART in Microbiology of Well Biofouling Lewis Publishers / CRC Press pages 435:137 - 280
[cxviii] Cullimore, D.R. and L. Johnston (2000) the Impact of Bioconcretious Structures (Rusticles) in the RMS Titanic: Implications to the Maritime Steel Structures. Technical Presentation, Annual Meeting, Society of Naval Architects and Marine Engineers, Vancouver, 9-1 to 9-16.

[cxix] Leggette, Brashear & Graham Inc (1997) well rehabilitation project funding agreement with American water Works Association Research Foundation, project 464 fifth periodic report appendix I Mosinee, WI 1 – 7 Table 1of2 BART tests; Appendix IV attached; Ames, IOWA, 5-6 table 3; Pinellas County, 3 – 4, & table; Appendix V Norwalk, CT. Birmingham CT 3 – 10 & table 4; Appendix VII, Rockland County, NY, 3, 5-6,; Appendix VIII, Houston, TX 3 – 4, Table 3; Appendix XI, UAB 1.3.3.12 – 13, SWWI 13 - 16

[cxx] PFRA / DBI / City of North Battleford (2000) Well Rehabilitation Report, 13 pages, four appendices see appendix three and pages 8 and 9.

[cxxi] U.S. Corp of Engineers (2000) Management of Injection and Extraction Wells at HTRW sites, engineering pamphlet 1110-1-27, section 2-9

[cxxii] U.S. Corp of Engineers (2001) Rehabilitation of Biofouled Injection and Extraction Wells at HTRW sites, engineering pamphlet 1110-2-27, 120 pages

[cxxiii] Johnston, L (2001) Investigation and Sustainable Management of Microbially induced biofouling in Porous Media using Electro-Motive Forces, M.Sc. thesis Royal Roads University 99 pages and 62 pages of appendices

[cxxiv] Thomas, Larry (2002) article to be published by the AWWA in Opflow that will include graphics on the SRB-BART and its application.