Historical Introduction

Microbiology, as a discipline, has moved through a number of different methods for the enumeration of bacteria. Bacteria have been traditionally perceived as being major inducers of a range of challenges in society from infection though spoilage to biofouling and system failures. Enumeration of bacteria has traditionally been culturally based commonly using agar-based culture media but more recent technologies employ chemical markers to detect and enumerate selected groups of bacteria[i]. These markers range from adenosine triphosphate[ii] (ATP, detects actively metabolizing cells, quantitative), to fatty acids, methyl esters (FAME, allows identification of many specific species, qualitative) and various factions of DNA[iii] and RNA (progressive technology mainly utilized for either refined qualitatively or semi-quantitatively to define selected microbial groups). While these tests do in different manners generate a high level of precision within narrow defined conditions, they commonly require sophisticated and often expensive laboratory equipment and highly trained staff to function effectively. Droycon Bioconcepts Inc in its mission statement defines its goal as "taking the laboratory to the field and not the sample to the laboratory". This ETV request specifically relates to the use of the HAB-BART system for the in-field detection of heterotrophic aerobic bacteria.

Biological activity reaction tests (BART™) were invented[iv] in 1986 and patented in 1991 as a means to detect specific groups of bacteria using activities and reactions detected during the incubation of the tester when:

- A selective culture medium diffused up from a basal crystallized pellet.
- Oxygen diffused downwards from the headspace gases.
- A reduction-oxidation gradient formed as the bacteria became active.
- Series of lateral environments were created within the tester
- There was preferential movement of bacteria within the sample to the more favorable environments being created within the tester.
- Population size was based upon the time lag to the first observation of a positive activity or reaction within the tester.

Heterotrophic aerobic bacteria (HAB) were one group of bacteria considered to be of importance in the monitoring of water and waste water quality[v] and a HAB-BART tester was developed by 1991 and entered commercial sales in 1993[vi]. While the BART testers for the iron related bacteria (IRB-) and sulphate reducing bacteria (SRB-) have now gone through the Canadian ETV process and have been verified (ETV 2002-31 and 2002-32 respectively), the HAB-BART system was under development to include as the primary tools: the tester, reader and a software program (BART-READ™, H4). This ETV request therefore justifies verification of these three items as components in the enumeration of heterotrophic aerobic bacteria.

HAB-BART testers have been used primarily within the water and waste water[vii] and environmental industries to monitor heterotrophic aerobic bacteria[viii] associated with water and water samples with high organic contents and the functioning of bioremediation processes. They were extensively used in the American Water Works Association Research Foundation (AWWARF) sponsored study of water well rehabilitation techniques mainly in the eastern states of the U.S[ix]. The U.S. Army Corp of Engineers (USACE)[x] also used the HAB-BART testers in monitor the effectiveness of rehabilitation techniques applied to injection and extraction wells on hazardous waste

sites. In Canada these testers were used to determine the effectiveness of well treatments on First Nations reserves to control health risks associated with heterotrophic bacteria and the HAB-BART showed similar data patterns to the standard heterotrophic plate count techniques employed by Health Canada[xi]. Additionally Canada Agriculture and Agri-Food used the testers to determine the level of microbial risks associated with two municipalities in Alberta and Saskatchewan. A joint project with Agriculture and Agri-Food Canada utilized the HAB-BART testers as a part of the process of monitoring[xii] the effectiveness of rehabilitating the water wells servicing the City of North Battleford, Saskatchewan[xiii]. Extensive field trials were also conducted in Egypt sponsored by the Canadian International Development Agency (CIDA) to examine the use of the HAB-BART tester along with other BART testers to monitor biofouling and health risks in the western region water well fields and also recharge wells along the river Nile (note this study was apparently successful but there has been no releases of documentation at this time). In all of the cases the HAB-BART tester was found to function well in field conditions generating data that correlated well with the known conditions for the wells and there was a mutual satisfaction with the functionality of the test.

To improve the understanding of the value of the time lag (TL) generated by the HAB-BART as well as the two reaction types (up, UP and down, DO) that indicate the direction in which the reduction front moves in the tester, two studies were initiated in Canada. First was a study in 2002 to 2003 at the University of Waterloo under the direction of Dr. Barbara Butler. Here direct comparisons were made between the TL and the data generated by the traditional heterotrophic plate count. Samples were obtained from the water treatment plant and distribution lines operated by the City of Espanola, Ontario. Tests were performed on the fresh samples as wells as at a local laboratory and again after twenty four hours (standard shipping time) at the University of Waterloo. The outcome of those studies forms a part of the verification of the claims. Second was a study conducted at the University of Saskatchewan that began in 2003 and concentrates on the link between TL and the ATP content of the samples. These samples are being taken primarily from various wells and soils associated with bioremediation activities. This study is still in its infancy.

Issues that have led to the development of the HAB-BART system include the following:

- Need to improve precision of data obtained relating to sampled material by reducing the errors associated with storage and shipping to a minimum by taking the laboratory to the sampling site and reducing post-sampling impacts on the data generated.
- Need to assure consistency in operator procedures through simplifying the testing process with a minimum effort level demanded of the operator and the test data acquired by a fully automatic monitoring system that operates with precision.
- Need to have a chain of custody that will allow recovery of archived test data in the event of a dispute or concern.
- Need to allow confident comparisons of data generated over time from samples taken from common or related sources.

The system causes all data to be stored in Excel spreadsheet compatible .txt files with the graphical information stored in Adobe Acrobat PDF format or files that can hold up to six sets of tester data. This allows the free exchange of generated data over the internet or through land/satellite links.

This request to ETV Canada Inc. includes the ability of the HAB-BART system to work with all water-based samples of environmental origin where there is a need to quantitatively and semi-qualitatively determine the activity of heterotrophic aerobic bacteria. Quantification is primarily using the time lag that is determined in seconds with the common range being from 500 to 432,000 seconds (or 8.3 minutes to 5 days). Semi-qualitative determination is limited to the detection of two activities that are generated by the aerobic heterotrophs (UP reaction) and those heterotrophs that are active under anaerobic conditions (DO reaction). It should be noted that the broad group of heterotrophic aerobic bacteria would be active in both reactions but would include strictly aerobic heterotrophs in the UP reaction and would be dominated by facultative anaerobic heterotrophs in the DO reaction.

ETV REQUEST

1. Definition of Claims for the HAB-BART system (version H4)

The following lists the specific claims for the HAB-BART system that are a part of the verification request. These claims relate to the use of the HAB-BART tester integrated with the HAB-BART reader (H4) and BART-READ software (version H4) only when connected to a Windows 95 or better computer (see also appendix I). These claims are made relating to unique features used in the operation of the HAB-BART system. Other aspects of the testing procedure relate to normal well recognized aseptic techniques and disposal practices that are commonly applied within microbiology. The connection of these three components therefore forms the basis upon which the following claims are made:

1. That the HAB-BART tester provides an adequate manner to allow the effective detection of activities for the various forms of heterotrophic aerobic bacteria.
2. Detection of heterotrophic aerobic bacteria is determined by the impact that these bacterial activities have on the culturing fluids within a HAB-BART tester that has been incubated after being charged with the water based sample.
3. The first observable impact created by these activities within the tester would be the observation of a reductive zone within the culturing fluids due to the losses of oxygen from that zone resulting from respiratory activities caused by the microorganisms in the sample. The delay in observation of that event forms the recorded time lag (in seconds) used to define the aggressivity / activity levels of the heterotrophic aerobic bacteria in the sample being tested.
4. Basic characterization of the heterotrophic aerobic bacteria can be determined by the manner in which the reductive processes associated with the decolorization of the methylene blue ("bleach front") moves up (UP reaction type) or down (DO reaction type).
5. Methylene blue, through its color changes being dictated by the oxidation-reduction potential at the site of measurement, provides a binary record of whether the local condition in the tester under examination is oxidative (blue) or reductive (colorless). Diffusion of the basal nutrient pellet and initial microbial activities can commonly cause the reductive state to appear yellow rather than colorless.
6. Population projections can be achieved using the time lag and converting this to the projected population using an equation installed in BART-READ (H4). This population is projected as predicted active cells / ml that are equivalent to colony forming units / ml and is displayed on the graphing screen from the moment of positive declaration.
7. In the operation of the testing procedure the temperature for incubating the testers is controlled by an isothermic block within the reader that is set to give a temperature range of $28 \pm 0.5^{o}C$. This temperature is within the optimal growth range for mesotrophic bacteria commonly found in the environment.
8. Temperature range for any given test is displayed graphically by BART-READ (H4) through the real-time graphing of the data and reflects the mean temperature together with the highest and lowest temperatures detected.

9. Limits for detection of heterotrophic aerobic bacteria would be one actively respiring cell in the 15ml sample. This would provide a maximum detection limit of one cell per 15ml, or 67 cells per liter.
10. The methodology as described will function in water and waste water samples having a salinity of less than 4% sodium chloride equivalents.

2. Conceptualization of the HAB-BART tester

This section addresses the unique nature of the BART tester as a culturing system for the detection of microbial activity. Through the seventeen years of development a number of concepts have been generated which remain to be published in a formal manner and so needs to be held confidential but these concepts may aid in recognition of claims given in section 1 and defended in section 3.

CONCEPT: that the BART tester invokes an environment confined by the tester that will be critically affected by the connectivity between that environment and the greater environment that is outside of the tester. Three domains are contemplated:

- **SEPARATED ENVIRONMENT** in which the sample contents of the BART tester is denied any linkages to the outside environment through the use of constraining impermeable barriers composed of seals and low permeability constructs.
- **LINEARIZED ENVIRONMENT** in which there is a linear connectivity between the sample contents of the BART tester and the outside environment through the capping structures while the tester environment is contained in all other directions. This linearized connectivity allows a "balance" to be achieved between the confined (tester) environment and the larger embracing environment.
- **INTEGRATED ENVIRONMENT** in which the barriers confining the sample in the tester do not prevent interaction between the sample and the surrounding environment. Here the barriers forming the tester do allow the movement (in a restrictive manner) of gases and water through, or around, the barriers.

It should be noted that the term "environment" used here is a relative term and does not exclude the impacts of outside environmental factors on the controlled movement of thermal and light energy and incidental forces such as those associated with generated or natural electromagnetic forces.

Basic domains supporting the BART concept as a defined "environment" in that the contents of the BART are at least partially independent of the surrounding environments include the following:

1. Enhancement of the BART tester environment by the addition of a dried specific culture medium allows the environment within the BART to become supportive for the targeted consortium of microorganisms of interest that may be in the sample under examination.
2. Selective culture of a particular consortium in a staggered manner in which the natural shifting in the environment within the restricted environment along with the products of intrinsic cultural activity causes a gradual shift in the dominant

organisms within the consortium as a result of the environmental changes within the environment inside the BART. This would create a condition where mutualistic supportive activities within the consortium are supplanted by competition leading to a distortion in the make-up and presentation of the consortium within the BART tester through the generated activities and reactions.

3. Optimization of the initial environmental balance within the BART environment provides a common starting point to the BART device system in which the initial restrictors to microbial activity are presented through the composition of the sample being examined.
4. Expression of the BART environment would therefore be a result of the presence of a suitable consortium that had not been suppressed within the sample by restrictive factors present within that sample (such as residual chlorine or toxic metals like lead, arsenic or copper). This expression would be a reflection of the cascading ability of the various members within the consortium to become active, create reactions and then become suppressed through the subsequent activities of other members of the consortium within the sample. The device system therefore involves a dynamic state in which mutualism precedes restriction as some members of the consortium rise to a dominant state involving the suppression of other members of the consortium.
5. End points in the BART environment are represented by detectable activities or reactions that occur after a period of time and are recognized as being significant to detection of a positive for the consortium being investigated. It is recognized that this end point would be influenced by a level of activity and reactions within the consortium and would materialize when the net interactions between the components that reach defined stages of development by recognizable end points This time lag would form such an endpoint and as such would therefore represent a body of activity involving mutualistic and antagonistic interactions between the living components of the consortium within the sample at that time.
6. Essentially the BART environment provides an ability to detect the activity level of the consortium within the sample being incubated and the organisms are moving through the phases of nutrient diffusion (as the medium in the floor of the tester dissolves and rises), interaction between the organisms and the physical and chemical nature of diffusing matrices including oxygen coming mostly downwards in the sample column in the tester, and then the generation of limitations resulting from the depletion of nutrients and oxygen (due to consortial activity) and the build up of restrictive end products being generated the activity and reactions of at least some members of the consortium.
7. Precision and interpretation is based upon the time lag to a recognized state within the HAB-BART environment (e.g., going reductive). This interpretation represents the time delay (lag) over which a complex of microbial cells achieved a given and recognizable metabolic state (the utilization of all of the local oxygen and creation of a reductive state). It is proposed therefore to consider that the time lag reflects the status of the number of active cells in the consortium under investigation with two cells could exist in one of two states:
    - ACTIVE in which there does not appear to have been any lag times before the consortium became active in the BART tester.

- STRESSED in which the time lag was delayed to such an extent that the consortium had to pass through an initial induction phase before it could become active.

From the investigations to-date it would appear that a time lag of 86,400 seconds would appear to commonly be the interface between consortia dominated by active, viable cells will thus have a shorter TL than those dominated by stressed, viable cells that would have a longer TL.

3 Supporting Evidence for the Claims as listed in Section 1

3.1 That the HAB-BART tester provides an adequate manner to allow the effective detection of activities for the various forms of heterotrophic aerobic bacteria.

Heterotrophic aerobic bacteria are defined as a group by their ability to exploit the degradation of organic materials as the main source of both energy and nitrogen and phosphorus rich nutrients. As a group they vary in requirements from species to species but collectively as a community possess the resources to utilize carbohydrates, lipids and proteins. The culture medium used for the crystallized culture medium that is rich in diverse carbohydrates, and proteins (proprietary LCP medium).

3.2 Detection of heterotrophic aerobic bacteria is determined by the impact that these bacterial activities have on the culturing fluids within a HAB-BART tester that has been incubated after being charged with the water based sample.

While many traditional cultural broth tests involve the determination of changes in the clarity of the medium due to turbidity resulting from suspended microbial growths within the culturing fluids, this test determines respiratory microbial activities that utilize the oxygen. Here it is the faster consumption of oxygen than can be compensated for by primarily downward oxygen diffusion from the head space in the BART tester that causes the depletion of oxygen concentrations leading to establishment of reductive conditions. This depletion through creating reductive conditions is then monitored colorimetrically using the methylene blue.

3.3 The first observable impact created by these activities within the tester would be the observation of a reductive zone within the culturing fluids due to the losses of oxygen from that zone through respiratory activities caused by the microorganisms in the sample. The delay in that observation event forms the recorded time lag (in seconds) used to define the aggressivity / activity levels of the heterotrophic aerobic bacteria in the sample being tested.

This HAB-BART test functions primarily through the determination of the focal site for the first significant respiratory activity that causes that specific site to become reductive as indicated by the decolorization of the methylene blue as it moves into a reductive state. Principles applied in this test follow the standard methylene blue reductase test that has been in common use in the dairy industry for the last eighty years[xiv] or more[xv]. While the use of methylene blue reduction to detect is well known in the dairy industry[xvi], different advantages of this reductive activity have not been so fully explored. The HAB-BART tester equates the delay (i.e. the length of the time lag measured in seconds) to the numbers of respiring cells within the sample being evaluated. Thus an inverse link is claimed to exist between the size of the active (respiring) cell population and the length of time (time lag) before reductive conditions can be observed. This premise has been widely applied in the BART technologies in the sense that, whichever test is being performed, the shorter the time lag then the greater the incumbent active population in the sample. Since the test estimates activity levels in a direct manner rather than cell numbers, time lag measurement is considered to relate more closely to the aggressivity of the heterotrophic cells in the sample rather than the number of cells. This test therefore records the active of the aggressive (respiring) bacteria within the sample and the subsequent extrapolation to a population is based upon that active population. Given that many cells within the sample may not be active because of a combination of: (1) inability to adapt to the culturing conditions within the HAB-BART tester; and (2) cells being in either a sufficiently stressed, or suspended animation, state that prevents activity occurring during the incubation period. Technically therefore the time lag can be related more specifically to the number of active (aggressive) cells in the population that are able to respond to the environments created by the culturing fluids incubating within the HAB-BART tester. This would mean that the more precise definition for the population projected should be “predicted active cells per ml (pac/ml)” rather than the traditional “colony forming units/ml (cfu/ml)”.

3.4 Basic characterization of the heterotrophic aerobic bacteria can be determined by the manner in which the reductive processes associated with the decolorization of the methylene blue ("bleach front") moves up (UP reaction type) or down (DO reaction type).

In the eight years of development of all aspects of the HAB-BART tester, these two reaction patterns (UP and DO) form the vast majority of the tests observed with only a small minority (<2%) giving an anomalous result that still remains detectable as a reduction event. Where replicate analysis has been performed on water samples, it has been found that the reaction pattern remains consistently the same indicating that this reaction is fundamental to the community of microorganisms present in said sample. Since the development of the HAB-BART tester there have been many occasions where it has been found that the UP reaction tends to occur on occasions when there is a dominance of heterotrophs that are growing primarily aerobically. Such events are likely to occur immediately downstream from major sites of oxidative microbial activity along a redox front. DO reactions, by contrast, appear to occur in waters dominated by facultatively and strictly anaerobic heterotrophs that are active under reductive conditions. Here the water sampled would be upstream from any redox front where heterotrophic bacterial activity can be expected to focus. In summary, UP reaction may imply that the biofouling is upstream from the sample site while a DO reaction would imply that there is likely to be a major biofouling site downstream from the sampled site. With the DO reaction, the activity associated with the water sample would be anaerobic since it would be occurring under reductive oxygen-limited conditions. Such activities tend to generate smaller organic daughter products such as the short chain fatty acids that move downstream to breakdown when conditions become oxidative as the water crosses the redox front. Here there would be magnification of the biological activity that would then often be associable with some form of biofouling[xvii xviii].

3.5 Methylene blue, through its color changes dictated by the oxidation-reduction potential at the site of measurement, provides a binary record of whether the local culturing conditions in the tester under examination is oxidative (blue) or reductive (colorless).

Methylene blue is well known as a redox (oxidation – reduction, ORP) indicator shifting from blue under oxidizing conditions to clear under reductive conditions. It is also known to be a biological stain but toxic characteristics against bacteria have not been extensively recognized. Since methylene blue is a critical integral part of the HAB-BART tester[xix], a 230 sample study was conducted to determine the impact of methylene blue (MB) on the activity of different groups of bacteria. To achieve this, the HAB-BART tester was used with and without MB (as the control). For the control it was not possible to detect the shift to reductive conditions since no indicator was added in the control. As an alternative method for detecting the start of growth, the visible generation of turbidity was used to mark the beginning of heterotrophic bacterial activity in the control. Data was drawn from three sampling points on a biofouling megacosm and the comparison of time lags (given in days) between the control and the MB standard format is given in Table 3.1.

Table 3.1, Impact of methylene blue on heterotrophic bacterial activity

Trial #	BART type	Mean TL	Standard deviation	Longest TL	Shortest TL	Number Samples
T1	Control	1.59	0.42	4	1	76
T1	HAB-BART	2.42	0.63	5	2	77
M1	Control	1.66	0.58	4	1	76
M1	HAB-BART	2.49	0.93	8	2	77
B1	Control	1.62	0.52	3	1	76
B1	HAB-BART	2.43	0.75	5	2	76

Note: each HAB-BART tester cap contains 0.31 mg of crystallized methylene blue.

There was a consistent difference between the control (averaging for the three trials a time lag of 1.62±0.51 days) and the HAB-BART (averaging 2.47±0.77 days). This difference of 0.85 days with an increased variability (of an addition ±0.26 days) may be attributable to the presence of methylene blue having an inhibitory effect on the active bacterial population except that the method for detecting activity was also different (the formation of turbidity in the control and the reduction of the MB in the HAB-BART). Given that the occurrence of turbidity almost always follows the development of reductive conditions (i.e. MB reduction in a HAB-BART precedes turbidity) then the probability is that MB does have an inhibitory effect on the heterotrophic bacteria that causes prolongation of the time lag. This effect would appear to be transitory since in all cases observed there is subsequent activity. It is therefore claimed that while the methylene blue does act as a transitory inhibitor to the activity of heterotrophic aerobic bacteria this impact is consistent resulting in a 52% average increase in time lag. There remains a risk that

there may be random impacts by the MB that could cause losses in precision. Multiple replication of the HAB-BART tester was undertaken primarily using samples from the various stages in the aerated lagoon treatment of municipal waste. A summary of the precision achieved in these trials is listed in Table 3.2.

Table 3.2, Precision of the HAB-BART tester using treated municipal waste water

Sample source	Number replicates	TL seconds (mean)	Standard dev.	Variability
Primary effluent	20	4,425	425	10%
Lagoon 2	8	33,035	890	3%
Tertiary effluent	19	37,468	963	3%
Tertiary effluent	20	43,516	1,400	3%
Final effluent	27	43,182	3,287	8%

This trial used the HAB-BART reader system and so the time lags were generated in seconds. For three of the five trials the standard deviations achieved were less than 4% of the mean value while the other two trials showed a variability of up to 10% of the mean value. There were no outliers that were greater 15% from the mean values indicating that the MB did not appear to cause greater variation in the time lags than may be expected from the normal natural variations between samples. Therefore while it is admitted that MB does have an inhibitory impact on the respiratory activities of the incumbent heterotrophic bacteria in the sample, it is claimed that the impact is universal and does not significantly affect the accuracy of the test method but simply delays the onset (recognition) of a positive declaration of the test through the generation of a time lag.

3.6 Population projections can be achieved using the time lag and converting this to the projected population using an equation installed in BART-READ (H4). This population is projected as predicted active cells that are equivalent to colony forming units / ml and is displayed on the graphing screen.

While it has been demonstrated above that the level of heterotrophic bacterial activity can be measured by the time lag to determinable reductive conditions, it does also by implication relate to the population of active cells that are present in the culturing fluids of the HAB-BART tester and introduced with the sample. Clearly the greater the number of respiring active cells then the faster would be the depletion of oxygen with the culturing fluids and the sooner a reductive condition would be established. Therefore logic would dictate that the greater the number of respiring cells then the sooner determinable reductive conditions would be established. This would mean that the greater the active population then the shorter the time lag obtained to reductive conditions. A demonstration of the relationship between populations obtained at various dilutions of three pure cultures of different bacterial species is given in Table 3.4.

Table 3.4 Influence of population size on time lag using the HAB-BART system for three different species of bacteria

Species	Regression equation	R^2	Replication
Pseudomonas aeruginosa (27853)	Y = -15397x + 179831	0.92	Three trials, 6 replicates each dilution to 10^{-12}
Escherichia coli (25922)	Y = -8933x + 104323	0.91	Three trials, 6 replicates each dilution to 10^{-12}
Klebsiella pneumoniae (13883)	Y = -5604x + 65351	0.98	Three trials, 6 replicates each dilution to 10^{-12}
Pooled data	Y = -10052x + 117183	0.54	Pooled data obtained from the above trials using pure cultures of specific species

Note: x represents $\log_{10}$ cell population in cfu/ml and Y represents the time lag in seconds to a positive detection of heterotrophic activity through the reduction of the methylene blue. Bracketed number is the ATCC strain number for the species in the same box.

For all three ATCC strains of heterotrophic bacteria, very good correlations were obtained between the time lag and the predicted cell populations obtained through dilution – spread plate analysis to determine the number of colony forming units / ml in the various dilutions used for the HAB-BART trials. Acceptable R^2 correlations were observed 0f 0.92, 0.91 and 0.98 using linear regression analysis.

However there were considerable variations in the interception with the y axis (where x was the population and y was the time lag in seconds). Intercepts varied from 65,351 (*Klebsiella pneumoniae*) to 104,323 (*Escherichia coli)* and 179,831 seconds (*Pseudomonas aeruginosa*). When the all of the data was pooled together then the R^2 value fell to 0.54 because of the variation between each of the species of bacteria when subjected to testing using the HAB-BART system. This poor correlation was a reflection of the different generation times for each of the species and their potentially different sensitivities to the methylene blue dye.

The premise that there is a definite link between the time lag and the population would therefore appear to be substantiated at the pure culture level with an acceptable ability generated to project cell populations by the time lag. However once the data for the three species was pooled then the precision was reduced mainly because of the different manners with which the three species responded to culture in the HAB-BART tester. Given that this variable response pattern is also likely to occur in natural samples then the value of the time lag information to project population size would appear at least partially compromised. Pure culture studies do not however reflect the real world conditions that would exist in samples derived from natural sources. Here the samples would include a mixture of species some functioning within communities while others operate independently. Placing such a mixture into the layered environments within a charged BART-BART tester means that there would initially be phases of: (1) relocation and adaptation; (2) competition and cooperation; and (3) dominant respiratory activity for the successful species / communities. This test terminates with the onset of reductive conditions and the establishment of a time lag. These three phases could all affect the rate at which the time lag was declared.

Time lag as a function is a direct reflection of that point in time when oxygen becomes depleted along either of the two lateral sensing light channels and is a product of oxygen depletion establishing locally reductive conditions. Experience has found that TL of less than 86,400 seconds (one day) follows a linear regression analysis pattern summarized as:

$$\text{Log}_{10}\,(\text{Pop}) = ((-5 \times 10^{-5}) \times (\text{TL})) + 11.144$$

In which Pop refers to the population expressed as pac/ml, and TL is the time lag (generated in seconds). This equation was based primarily on the pure culture studies with comparisons made to agar spread plate analysis of various dilutions. It was found the TL data generated at greater than 86,400 seconds showed a different correlation and the regression equation had to be modified to:

$$\text{Log}_{10}\,(\text{Pop}) = ((-2 \times 10^{-5}) \times (\text{TL})) + 8.699$$

Here, there appeared to be a slower rate of reductive activity associable with the respiratory activity of the cells. The reason for this remains unclear but possibly links to the relationship of the active cell volume to total culturing fluid volume to achieve a maximum in metabolic activity. Greater dilution possibly limits the metabolic efficiency of the individual active cells thus reducing the rate at which respiratory activity and oxygen depletion can occur. Populations are calculated using the $\log_{10}$ base and then converted to the arithmetic form as cap/ml.

3.6.1 Confirmatory Studies, University of Waterloo

In the summer of 2003, Dr Barbara Butler of the University of Waterloo started an evaluation of the suitability of using the HAB-BART system for the monitoring of heterotrophic aerobic bacteria as a replacement for the heterotrophic plate count. This study was a joint study with the City of Espanola and BOD Consulting Inc. sponsored by Ontario Ministry of Environment and Energy with all of the systems provided at no cost by DBI. Focus for the study was on water quality issuing from the treatment plant and moving through pipelines in the distribution system. Two major questions to be addressed related to the issue of sample handling and storage, and also the suitability of the HAB-BART system to reliably detect and enumerate heterotrophic bacteria with accuracy at least comparable to the plate count (HPC) method. At this time the final report is being prepared and this ETV verification will be modified in this section to address the findings of that report. By initial conversations with BOD Consulting Inc. indicate the following findings that are relevant to this application:

a) HAB-BART system achieved similar patterns of results as the HPC with improved ability to detect lower populations (due in part to the greater volume of sample used and the removal of the need to dilute).
b) HAB-BART reader functioned effectively in all of the conditions established (in the treatment plant, local consultant's laboratory and the University of Waterloo laboratories).
c) Storage and shipping of samples had a deleterious impact on the precision of the testing methods indicating the superiority of be able to test the sample effectively at the site of sampling.
d) Generation of a record of the testing procedure was considered a major advantage for archival reference when various management techniques relevant to the water quality were being applied.
e) Chain of custody could be created that would ensure improved management and accountability.

~~3.6.2 Confirmatory Studies, University of Saskatchewan~~

~~From 1991 to 1996, cooperative studies were undertaken with the University of Western Ontario to determine factors influencing plugging in the drains of sanitary landfill operations. Under the direction of Dr Kerry Rowe, studies were conducted on the various bacterial communities that impacted these plugging processes. At that time, only initial limited comparisons have made between pooled time lag data from BART testers and ATP at two temperatures. Linear correlations were obtained for both temperatures but there was not enough data to statistically support the nature of this relationship. Dr Ian Flemming now at the University of Saskatchewan has now begun an independent study of the nature of this linkage. In the summer of 2003, forty one samples were taking from crude cultures and impacted environments and both the TL (using the HAB-BART system) and the ATP were immediately determined using non-standard ATP testing procedure. A sigmoidal relationship was established of sufficient confidence to proceed now to a defined~~

~~scientific study that is currently underway at the university using standard ATP test procedures on fresh samples. A crude relationship between TL and ATP (expressed as relative light units, RLU) is given in Table 3.5 and verifies that need to proceed to a defendable determination of these relationships.~~

~~Table 3.5, Provisional Relationship of TL to ATP using the HAB-BART system~~

~~TL (seconds)~~	**~~ATP (RLU)~~**
~~1,000,000~~	~~4,000~~
~~100,000~~	~~20,000~~
~~10,000~~	~~600,000~~

~~This section of the ETV claims will be up-dated by December 15, 2003 to further support the claims as described.~~

3.7 In the operation of the testing procedure the temperature for incubating the testers is controlled by an isothermic block within the reader that is set to give a temperature range of 28±0.5°C. This temperature is within the optimal growth range for mesotrophic bacteria commonly found in the environment.

Incubation temperature selection is a key to the determination of the activity level observed using the HAB-BART system. In addressing this issue, the system provides a standard incubation temperature (selected to be 28±0.5°C) but is also adjustable to other temperatures through software selection (see section following the justification of the standard selected temperature. Common temperatures used for the incubation of mesotrophic heterotrophic bacteria in practice ranges from 20oC through to room temperature, 25°C, 28°C and 30°C with normal body temperature for humans (37°C) also being used. This latter temperature is commonly used for the incubation of potential warm blooded pathogens but may be too high for many mesotrophic heterotrophs. Evaluations were undertaken to determine the most effective incubation temperature for the heterotrophic aerobic bacteria. This was done using samples from the Regina Waste Water Treatment facility (Table 3.6).

Table 3.6 Effect of incubation temperature on time lags for treated effluent

°C	average	st dev	high	low	% var
5	52,085	4,819	55,588	46,590	9.3%
11	16,835	4,006	22,808	14,273	23.8%
22	7,721	1,134	9,310	6,788	14.7%
25	7,256	1,042	8,526	6,086	14.4%
37	6,659	751	7,658	6,032	11.3%
45	7,302	1,067	8,354	5,818	14.6%

In this study involving ten replicate studies, it was found that the time lag appeared to be reduced in the incubation temperature range of 22 to 37°C with the variability falling from to the less than 14%. However this study indicated that there was a need for a more precise evaluation of the incremental effects of incubation temperature on the time lag. Using a thermal gradient incubator, a series of studies were conducted (Table 3.7) examining the variability of time lags generated at 22 and 37°C in comparison to 28°C using tertiary effluent as the source of heterotrophic bacteria. This included eight replications in four trials with the time lag shifted to a factorial value of the TL data generated at 28°C.

While the shortest time lags occurred at 37°C (0.74 to 0.98 of that found at 28°C) with a variability of less than 3% concern was noted from the relationship variation with 28°C data. In trials 1 and 2 the TL was significantly shorter at the higher temperature. However in trials 3 and 4 the difference was marginal (2% TL difference in trial 3 and 11% in trial 4). This indicated that the use of 37°C as a standard may be less precise for measuring mesotrophic heterotrophs that have optimal temperatures within the lower mesotrophic range (25 to 30°C). 22°C gave

time lags that were 60 to 70% longer than 28°C with similar variabilities. This trial indicated that 28°C should be the preferred temperature.

Table 3.7, Factorial variance at three incubation temperatures on time lags in four trials using tertiary effluent

Trial #	37°C	28°C	22°C
1	0.74 (±2.9%)	1 (±1.8%)	1.6 (±5.6%)
2	0.71 (±2.8%)	1 (±7.9%)	EF
3	0.98 (±1.6%)	1 (±3.2%)	1.7 (±2.4%)
4	0.89 (±2.9%)	1 (±2.8%)	1.6 (±3.2%)

Note that the mean value for the 28°C replicates was used to calculate the factorial variation for all individual samples at all three temperatures.

A trial using six replicates was conducted using primary influent and effluent as well as tertiary effluent to examine the longer termed impact of the incubation temperature on the time lag variability (Table 3.8). Note that one trial (EF) was the subject of an equipment failure.

Table 3.8, Ten week investigation of affect of incubation temperature on TL

Source	37°C	28°C	22°C
Primary Influent	3,924±471 (12%)	3,685±288 (7.8%)	4,992±306 (6.1%)
Primary Effluent	8,952±805 (9%)	7,833±30 (0.4%)	10,668±904 (8.5%)
Tertiary Effluent	34,590±660 (1.9%)	47,891±942 (2%)	78,343±3,559 (4.5%)

In this trial it was noted that for the primary samples the time lags were shorter at 28°C rather than 37°C with less variability and yet the time lags were much shorter than those obtained at 22°C. This variability in the TL at 37°C may be a reflection of the activity levels of the heterotrophic bacteria that have higher optimal temperatures in the range of 37°C and could be related to potential warm blooded pathogens and commensals.

Given that tradition literature commonly recognizes 28°C as being a suitable temperature for the culture of non-pathogenic mesotrophic heterotrophic bacteria and that the laboratory studies summarized above also demonstrates the efficacy of using that temperature it has become the adopted standard incubation temperature for the HAB-BART reader.

It should be noted that under special circumstances the incubation temperature can be adjusted within the range of 8 to 40°C using the BART-READ (H4) software provided that the reader remains at least 5C° above the background temperature of the place where the reader is installed. This would mean that on some occasions the

reader would have to be installed in a refrigerated temperature controlled room or chamber (such as a refrigerator).

One aspect of the HAB-BART system is the potential to exploit two incubation temperatures for the test to determine what fraction of the activity within the sample is associated with potential warm blooded pathogens (having a greater health risk factor) rather than with the normal saprophytic mesotrophic heterotrophs. Health authorities assume that an equal or greater population being determined by incubation at blood heat rather below 30^{o}C would indicate a greater health risk. Water supplies having a higher presence of this activity or considered to have an increased health risk and restrictions may be placed on water usage by the impacted community. In such events, the claims are limited for the calculation of predicted active cells / ml (pac/ml) using TL data generated at 28^{o}C only. Time lags obtained using different operating temperatures cannot be used to calculate pac/ml and should only be used in a direct comparative manner with TL data generated on other occasions.

3.8 Temperature range for any given test is displayed graphically by BART-READ (H4) through the real-time graphing of the data and reflects the mean temperature together with the highest and lowest temperatures detected.

For the user of the HAB-BART system, the temperature of each pod is displayed on the chart graphing the data from the pod shown in the main graphing screen. This temperature is the average of the temperature data gathered from the isothermic block for that time the pod has been testing the in-place tester. Data generated includes the average, the lowest and highest point value generated during the testing period for that pod. It should be noted that in the event of the operator starting up a test before the isothermic block has reached the correct temperature range then the low value would be the first temperature value after the test is started. The highest value represents the maximum recorded value for the isothermic block during the test run to the point that the last data point was received.

BART-READ (H4) software includes a temperature modification program that allows the user to reset the incubation temperature of the block during the first 15 seconds that the reader is powered up. Once programmed, the reader will use the newly installed temperature settings to run future tests. This will continue until such time as the temperature is reset again.

3.9 Limits for detection of heterotrophic aerobic bacteria would be one actively respiring cell in the 15ml sample. This would theoretically provide a detection limit of one cell per 15ml, or 67 cells per liter.

Given that 15ml of water-based sample is used in this test procedure there would need to be at least one active bacterial cell capable of respiration within the culturing fluids of the tester in order to be able to eventually create a positive time lag through the generation of reductive conditions. It should be noted that higher numbers of cells than 1 cell per 15 ml or 67 cells per liter are likely to create a positive time lag that would relate to the number of cells that became active within the incubating tester conditions during the test period. This therefore forms the theoretical premise for the claims that the length of the time lag (in seconds) is inversely related to the number of active cells within the sample being tested.

Appendix A, Functional specifications

A.1, HAB-BART reader, functional characteristics

1. Two active light scanning pathways in each pod monitoring a HAB-BART tester. Each red light is turned on for a minimum of 100 milliseconds prior to the detection of the light received by the phototransistor. This sequence is repeated every second to gather a sorption value of between 1 (low sorption) and 255 (high sorption).
2. All programming is burnt into the EPROM using C++ language. The objective is to allow routine detection of the sorption of light along the two standard pathways for each followed by interpretation for a possible positive result determined by comparison to a given threshold sorption value based on a standard factorial shift from the original value. The first confirmed positive result forms the database for the determination of the TL and calculation of the HAB using a standard equation. The order in which the shift in sorption is detected forms the basis for declaration of either a UP or DO reaction.
3. The light source used is a standardized red (660nm) led light that is aligned directly at the center of the phototransistor. It forms a light beam that has an initial emitted diameter of 3mm but extends outwards as a cone with an angle of extension of 30^{o}.
4. Data is down loadable through a serial port directly to a Windows 95 or better computer that is running BART-READ (H4) that is connected by a serial cable.
5. The reader has a backlit LCD screen that permanently displays the status of all six of the pods. These pods are arranged in two rows of three to allow six HAB-BART tests to be run at the same time. This LCD display devotes three lines of twenty characters to each row of triplicate samples being tested. Displayed on these rows is the status for tests as either: running (R) or the TL which is declared in the event of a positive detection. A negative is declared at the end of the testing period set at five days.
6. The module contains an isothermic aluminum block into which the six pods are seated. This block is heated using a 110v. AC heater rod that is controlled to maintain the insulated block at $28\pm0.5^{o}C$.
7. To power the module, 110v AC line (2 amps) is used. This is split 110v AC (for the heater) and 5 and 12 volts DC to operate the temperature control and hardware incorporated into the module. A rechargeable battery is incorporated into the reader to allow the ongoing operation of the 5 and 12 volt circuits in the events of a power outage. The casing for the module is powder coated aluminum and is grounded to reduce the risk of static charge compromising the tests.
8. All six pods are constructed using injection moulded foamed ABS with pre-aligned positioning of the two lateral channels for the red LED lights and the associated phototransistors.

9. To start up a test, the HAB-BART tester is inserted into one of the pods and the test is automatically started when the tester is detected by the upper light channel.
10. Testing sequence is at one-second intervals for each of the two light pathways with a minimum of a 100-millisecond gap between emissions from each of the lights (to avoid false light readings by the phototransistor).
11. Declaration of a positive detection is determined when there is a recorded shift in the sorption of the light that falls outside the normal distribution pattern for a negative and into the pattern that would be taken to indicate a positive detection of a reaction pattern. This is taken to be a decrease of 20% from the initial sorption reading in light output on a scale limited to 255 units.
12. Reader needs to be powered up at least one hour before the commencement of any testing to allow the isothermic block to reach suitable incubation temperatures. It should be noted that the reader should always be set up in a room or temperature controlled chamber that is at least $5C^{o}$ lower than the proposed temperature for the reader to assure precision in maintaining the temperature at within the operating range of $\pm 0.5^{o}C$.
13. Calibration of the light channels is software controlled by the EPROM program that allows adjustment to resisters controlling both the emitters and detectors.

Production and specification of the HAB-BART reader is subject to the standard company internal ISO standards and specifications.

A.2, HAB-BART tester, specifications

Production and specifications of the HAB-BART tester is governed by a series of company internal ISO standards and specifications.

A.3, BART-READ software

Production and specifications of the BART-READ software is governed by a series of company internal ISO standards and specifications.

DROYCON BIOCONCEPTS INCORPORATED

User Manual for the Prototype HAB-BART System™

FOR THE DETECTION OF HETEROTROPHIC AEROBIC BACTERIA (HAB)

HAB-BART Tester™ And HAB-BART Reader™

September 24, 2003

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Notice to the Reader:

HAB - BART™ is the trademark applied to the patented concept for the determination of the heterotrophic aerobic bacteria (HAB) in water and waste water. The claims for the effectiveness of the test are restricted to these processes only. Where the HAB-BART test is applied to samples other than water then the data gathered may not be effectively interpreted using the standard technique described in this manual.

HAB-BART tester™ is the trademark applied to the patented concepts employed in the BART test apparatus that allows a 15ml sample to be used and a time lag obtained that relates directly to the HAB of that sample. This time lag is measured in seconds to the confirmation of a reaction that is detected when the medium in the BART tester becomes reductive. Here it would be claimed that the shorter the time lag then the greater would be the HAB population.

HAB-BART reader™ is an electronic device that allows the detection of the time lags for up to six HAB-BART testers. The results are displayed on-screen in seconds that may be converted from a provisional table to heterotrophic aerobic bacterial population. Note that the reader is set up to encourage triplicate testing by the testing channels being set up as two lateral rows of three test pods into which the testers are placed. The reader operates on 120 volts AC that should be supplied using an uninterrupted power supply to assure the data is still gathered during any power outage of moderate duration. To improve precision the reader incorporates an incubator that maintains the testers at $28 \pm 0.5^{o}C$.

HAB-BART system™ refers to the total package for undertaking the determination of the HAB using the HAB-BART tester and reader in combination with BART-READ (H4) software.

Executive Summary

This document forms the basis of the HAB-BART system™ protocol as an alternate testing procedure to the standard heterotrophic bacterial spreadplate methods.

The HAB-BART system determines the HAB through the aggressivity of the bacteria that are active in the original sample. This aggressivity, measured by the time lag to the detection of activity, can be directly linked to the amount of bacterial metabolic activity occurring in the sample. Here, the greater the aggressivity in the bacteria (shorter time lag) then the greater the heterotrophic aerobic bacterial population in the water sample. The HAB-BART system uses a different method to measure HAB compared to the standard spreadplate methods but offers distinct advantages that, while achieving the same goal, estimation is made of the potential level of activity (aggressivity) of the heterotrophic aerobic bacteria in the sample

Differences between the two tests can be summarized as:

- Interpretation: The HAB-BART tester determines the aggressivity of the heterotrophic aerobic bacteria by the time it takes for the resident population in the sample to convert the oxidative state in the tester to a reductive state through the consumption of the oxygen that saturates the medium at the start of the test. The traditional test evaluates the number of colonies that grow in an agar medium as being directly related to the numbers of cells (colony forming units) that can be counted after a defined incubation period.

- Dilution: Dilution is an inevitable part of the procedure for the spread agar plate techniques in order to assure a statistically significant number of countable colonies at one particular dilution. For the HAB-BART, dilution is not required since the volume is set for the test at 15ml of the original sample with the size of the bacterial population recognized through the time lag to the point when the test medium becomes reductive (methylene blue turns from blue to clear).

- Incubation temperature. For the HAB-BART 28±0.5^{o}C is recommended since less variability has been determined in the time lag for the HAB-BART tester at that temperature range and an incubator (isothermic block) is built into the HAB-BART reader to facilitate the testing.

- Economics: The traditional spread plate techniques involves a significant amount of technician time to prepare dilutions, conduct the work under aseptic conditions and prepare the plates for the testing, and subsequently for performing the colony counting and interpretation to a predicted number of colony forming units. For the

HAB-BART system, the technician's time is reduced to less than 5 minutes per individual test when it involves the use of the HAB-BART reader. The HAB-BART system would therefore have the potential cost savings though using the system.

- Replication: The recommended replication is minimally duplication with triplicate samples preferred. In the HAB-BART system triplication of the test is highly recommended to assure better precision.

- Sample storage times: This should be restricted to less than two hours following the standard EPA procedures and preferably as short as possible. It is recommended that storage should be at room temperature.

* In the HAB-BART system, the data is generated as a time lag measured in seconds and is presented in that format on the reader. However when the reader is connected via a serial port to a computer then BART-READ (H4) does allow the computation of the predicted active cells/ml (pac/ml) that would be considered equivalent to colony forming units / ml (cfu/ml).

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1.0 Theoretical Considerations

1.1 The BART™ Concept

BART stands for the patented biological activity reaction test and is applicable for selected groups of microorganisms. Two of the BART testers have now received environmental technology verification from ETV Canada Inc for the IRB (iron related bacteria) and the SRB (sulfate reducing bacteria). Selection of each particular bacterial group is achieved by the use of selective culture media crystallized in the tester. The location of the bacterial activity is influenced by the formation of a reduction-oxidation gradient in the BART tester under test conditions. As the name (BART) implies, the test detects biological activity by looking for activities and reactions specific to the group of microorganisms being determined. Activities relate to growth events such as the formation of clouds, slimes, and gels. Reactions relate to the manners in which the microbes interact within the BART tester during incubation. These reactions may take the form of color changes, generation of gasses, and precipitants. The unique nature of the BART test makes it very different, and in some ways superior, to traditional techniques. This is partly because of the use of the undiluted sample that contains the indigenous microbes still within their natural environment and remaining at their intrinsic aggressivity (activity level). As a BART-based procedure, the testing procedure itself minimally intrudes into that environment during incubation thus allowing faster generation of activity and reactions. Major intrusions relate to the diffusion of nutrients upwards, oxygen down wards and the establishment of a redox front within the liquid sample column.

BART testers therefore use a unique system for encouraging the microbes to be detected during the test. First, there is normally no dilution of the sample prior to testing that would cause stress for the intrinsic bacteria in the sample. Second, the sample becomes exposed to a variety of different habitats within the tester that may be suitable for the different bacterial communities within the sample. Third, the microbes that do become active in and/or react with, the selective conditions created within the BART tester designed are those specific bacterial groups for which the test was designed. These selective conditions are created using two devices: (1) a floating ball, floating intercedent device (FID) restricts the entry of oxygen into the sample below; (2) a crystallized deposit of selective culture nutrients in the bottom of the tube encourages the activities and reactions by a specific group of microbes. Using the first (FID) device restricts oxygen entry primarily to that diffusing around the FID ball. This encourages the oxygen requiring (aerobic) microbes to grow close to, or around, the surfaces of this ball. These aerobic bacterial activities will use all of the oxygen diffusing down so that the sample further down becomes devoid of oxygen (anaerobic). This means that the volume underneath becomes suitable for the growth of anaerobic microbes that do not require oxygen. A single BART tester therefore provides a series of stratified environments that are aerobic (oxidative) above and anaerobic

(reductive) below. Essentially this becomes a reduction-oxidation gradient with a transitional zone (redox front) in the middle that focuses the growth, activities and reactions for the various bacterial communities that may be in the water sample being tested.

The key to determining the presence of the different groups of microbes is the crystallized selective medium attached to the floor of the BART tester. Upon charging the test vial with the water sample, this medium will begin to slowly dissolve upwards into the water column. As the medium dissolves, a series of chemical diffusion fronts become established and move slowly up the water column in the BART tester. The location of the growth within the test vial gives an early indication of the type of microbes involved. Activity in the base of the BART test would tend to suggest facultative or strictly anaerobic organisms while activity at the top around the ball is more likely to be aerobic. Often the activity may center along the diffusion front formed by the dissolving crystallized medium.

1.2 HAB-BART tester, theoretical

Given that the BART tester presents a whole range of environments within which the microbes can grow, the key to selectivity becomes the form of the crystallized culture medium that is in the tester. It is this factor that causes different communities of microbes to become active and, hence, become detectable. The BART tester therefore gives an indication of the types of microorganisms present in the test sample through the reactions and activities observed. Determination of the size of this population is achieved through the aggressivity of these active bacteria in the test vial. Aggressivity is determined by the speed (length of time for the time lag) before first observation (in the case of the HAB-BART tester then this would be a reductive condition arising from the conversion of the methylene blue from its blue oxidative state to the clear reductive state). The shorter the time lag to these initial confirmation then the more aggressive the bacterial population and the greater the active cell population would have to be. To determine heterotrophic aerobic bacteria using the HAB-BART system, the following parameters are critical to the effective detection of the heterotrophic aerobic bacteria:

- The medium is a very broad spectrum medium that has been found through twelve years of practical application to be the most sensitive to the widest spectrum of bacteria that are able to grow on this medium.
- Precision in determination is achieved using the redox indicator (methylene blue) that shifts from a blue (when oxidized) to a clear color when the cultured medium becomes reduced. This shift when first detected generates the time lag (TL) in seconds that is taken to directly relate to the aggressivity of the active bacteria within the test vial.

- Measurement of the TL event is achieved using the changes in sorption of a red (660nm) light pathway from high (blue oxidized) to low (clear reduced) sorption with sorption units (s.u.) moving from >180 down to less than 50. It should be noted that the standard critical point for the declaration of the TL is when the s.u. has declined by 20%.
- Population size for the heterotrophic aerobic bacteria is linked directly to the aggressivity of these bacteria as defined by the time lag length (in seconds) to the creation of the first localized reductive state.

The primary premise is therefore that the HAB-BART would detect the oxygen demand through the aggressivity of the intrinsic bacteria naturally present and active in the water sample being tested.

1.3 The HAB-BART Tester, concepts

The HAB-BART tester format revolves around the basic premise that the heterotrophic aerobic bacteria in the water sample may be effectively stimulated to become active by virtue of the variety of environments that are created by the contradictory diffusion of oxygen (downwards) and the enriched selective nutrient medium (upwards). This medium is saturated with oxygen at the start of the test procedure so that the focal sites for the first depletion of oxygen within the tester is a reflection of those sites where the bacteria are most actively consuming the oxygen and exceeding the supply of oxygen to that site. The more aggressive the HAB population then the more rapidly these reduction sites are detected within the tester and can be conveniently detected using the HAB-BART reader. The concept therefore is to use oxygen depletion through respiration as the prime determinant of the size of the active (aggressive) bacterial population. This differs from the traditional spread plate techniques in which the active cells have to be able to form recognizable colonies within, or upon, the agar medium in order to be enumerated. The vulnerability in the use of the colony forming method for detecting and enumerating the number of bacteria is that restrictions are created at least in part by the ability of the bacterial cells to actively grow to an extent that allows visible colony-like structures to form on, or within, the agar medium. These restrictions can become exaggerated by the nature of the bound water in the agar, effects of the highly oxidative surfaces to the agar, and the extractability of the nutrients from the agar medium to support growth.

Requirements for dilution are an integral part of the methodologies for measuring heterotrophic aerobic bacteria based upon the spreadplate cultural methodologies. Of these dilutions only a limited number will be useable for the determination of the bacterial numbers while the others would be either to numerous to count (too low a dilution), or too few to count (too high a dilution). For the HAB-BART tester, dilutions are not needed since the time lag (in seconds) effectively indicates the level of bacterial activity in the sample being tested.

1.4 Application of HAB-BART Principals to Monitor Population Size

The concept behind the patented HAB-BART system is based on the fact that a multiplicity of environments is established as stratified layers within the incubating tester. These extend from reductive (base) to oxidative (surface) lateral zones through which the selective nutrient medium gradually diffuses upwards. These environments are created by the admission of the 15ml water sample to be tested thus stimulating the activities of some of the intrinsic bacteria within the sample. In the HAB-BART system concept, the reaction of significance is the shift in the color of the methylene blue in the water from an oxidative (blue) state to a reductive (colorless) state. This test starts out in a highly oxidative state since the fluid sample in the test is saturated with oxygen by the (FID) ball rolling up and down the test vial as the tester is inverted five times. This oxygen comes from the head space atmosphere as it mixes with the sample. At the same time, this agitation also mixes the methylene blue (present in a sterile crystalline form inside the cap of the tester) evenly throughout the water. Bacteria and other respiratory microorganisms within the sample can now utilize this saturated oxygen in the water for the oxidative degradation of the organics in the sample. The more aggressive these bacteria are, then the shorter the TL before the methylene blue moves from its oxidized form to the clear, reduced form. Commonly this reduction occurs in the base of the test vial first and rises upwards. This is known as a bottom up (UP) reaction. As this reduction front rises (clear underneath / blue above) it is detected at the 14.5mm level above the base by a lateral red light channel. The down (DO) reaction and can commonly occur in water samples having a high population of microorganisms capable of anaerobic activities. In this case, the DO reaction would be detected first by the upper lateral red light pathway established at the 29mm level above the base of the tester. TL is then taken to be the first TL detected which would be in the lower light channel in the event of an UP reaction and the upper light channel in the event of a DO reaction.

The fundamental premise in the HAB-BART test is that the heterotrophic aerobic bacteria in the water sample primarily reflect the respirable organic substrates present in the sample and that this is then reflected in the aggressivity (activity) of the indigenous bacteria within that sample. In other words, the higher the active population of heterotrophic aerobic bacteria, the greater the available organic substrates, then the more aggressive the indigenous bacteria and the shorter the TL obtained using the HAB-BART tester.

In the HAB-BART test the TL is obtained by a change in the sorption of two red (660nm) light path being beamed laterally though the test vial at 14.5mm (for an UP reaction) and 29mm (for a DO reaction) above the base of the tester. By nature of an example using municipal wastewater, such as the primary effluent, in the UP reaction this shift to a reductive condition begins at the base of the water medium in the tester. It then moves upwards to reveal a clear to yellow colored medium rising under the blue colored medium above. This forms into a bleached front that gradually rises until it is above the equator of the ball floating in the tester. The reverse event occurs in the DO reaction with the initial reduction occurring just below the floating ball. It should be noted that the surface layer of

the culturing liquid sample around and above the ball generally remains blue throughout the test period. TL is therefore a measure of the time from the start of the test (charging of the test vial with the liquid sample) to the bleach front moving upwards or downwards passed the lower or upper red light pathway causing a change in the sorption. This change in sorption is measured using a phototransistor positioned across the tester within the red light pathway. Here, the bleach front is signaled by a radical change from a low light signal being received by the phototransistor (due to sorption by the oxidized form of methylene blue) using a scale from 0 (no sorption of the light) to 255 (total sorption of the light). The first receipt of greater than 20% reduction in sorption due to reduction of the methylene blue when detected by the phototransistor sensor is used to determine the TL. This downward shift in sorption is due to the relatively unimpeded passage of red light through the medium that is shifting from an oxidative (blue) to a reductive (clear) state.

TL (measured in seconds) now becomes the prime measure of the aggressivity (activity) of the heterotrophic aerobic bacteria in the water sample being tested. The theory is that the aggressivity of the bacteria links directly to the numbers if cells within the tester. It is therefore proposed that effectiveness of the HAB-BART tester ability to determine the heterotrophic aerobic bacteria is most directly linked to: (1) the amount of organics that, through degradation, create an oxygen demand, (2) there is a direct theoretical link between TL and the aggressivity of the heterotrophic aerobic bacteria in the sample being tested, and also (3) the numbers of active heterotrophic aerobic bacteria inherent in the sample. In this premise there are a number of factors of importance. These relate to the quality management of the test procedure and include:

1. Samples must be tested using the HAB-BART system within two hours of the sample being taken and that the sample must have been kept at room temperature during that time to allow the bacteria in the water sample to be tested time to adapt. Prolonged storage is likely to impair the aggressivity of the bacteria and so give more variable and often extended TL.
2. Incubation will occur within the reader, indicating $28 \pm 0.5^{o}C$.
3. All testing would be conducted would include the HAB-BART reader. Each reader contains six pods in each of which a HAB-BART tester can be tested simultaneously with the TL determined through the routine scanning of the tester every second for the occurrence of a bleach front until a positive determination is achieved. Two lateral red light pathways set at 14.5 and 29mm levels above the base of the test vial perform this scanning. TL is determined by the time lag to the first detection of a significant decline (>20%) in sorption due to reduction occurring at the light pathway. Once this sorption shift is detected the TL, in seconds, is displayed.
4. The HAB-BART tester contains an adequate reserve of chlorine neutralizer that is capable of removing up to 5,000 ppm of residual chlorine. Chlorine residuals, when present, may not therefore be expected to directly interfere with the functionality and accuracy of the test. Furthermore, the aggressivity of the bacteria within a sample is not adversely affected by any residual chlorine in the test sample.

1.5 Theoretical Justification, HAB-BART™ Technology.

Accurate determination of the respiration rate (i.e. the rate of free oxygen consumption) is determined through the time that elapses before there is a positive reaction to create the time lag (TL) in seconds. This then is the essence of the rapid determination of heterotrophic aerobic bacteria in a water-based sample since they are able to biodegrade or consume organic matter in water bodies as their source of energy primarily using the available oxygen in the culturing fluids as the dominant electron acceptor. By far the majority of these heterotrophs function most effectively under aerobic conditions.

Most unique of the features in the HAB-BART tester are the addition of a diffusible specific enriched nutrient medium (from the base of the tester) and methylene blue (as the redox indicator dried in the cap of the tester). While there remains free oxygen at any site within the culturing fluids, the methylene blue dye in the liquid medium remains blue. As soon as all of the oxygen has been consumed by bacterial respiratory activity at that site, then the methylene blue shifts from its observable colored form to a colorless form. In other words, in the HAB-BART tests, when the liquid medium turns from blue to colorless then the heterotrophic aerobic bacteria have been sufficiently aggressive to have "respired off" all of the oxygen at that site. Note that the dried methylene blue present in the cap of each tester is dissolved into the water-based sample by inverting the HAB-BART five times up side down. During this process the FID ball traverses up and down the test vial ten times. This allows for a head space air to saturate the turbulent liquid sample with oxygen to saturation.

Methylene blue is a basic dye that can bind readily to the negatively charged microbial cells. Traditionally, this dye has been used to stain microbial cells. The important property of methylene blue dye is that it changes from its original blue color in the oxidized state, to a clear state in the reduced state with the progression in oxygen consumption. When methylene blue is added to a liquid medium with respirable organic concentration, electrons are transferred to the dye causing it to become reduced and the blue (oxidation) color disappears. The rate at which this happens is dependent upon rate of the microbiological respiratory activity

With the UP reaction the methylene blue solution bleaches (decolorizes) from the bottom up. The bleached zone underneath may appear to be either clear or clouded. In the latter case, the medium tends to have a light to medium yellow color. Rarely does the bleaching extend above the equator of the ball so that a blue ring will normally remain around the ball with a depth of 1 to 5 mm below the surface. With the DO reaction, the bleaching usually forms just below the FID ball in a zone 32 to 42mm above the base of the test vial. The lateral red light channel placed at 29mm with a width of 3mm is able to detect this DO reduction zone as soon as it has formed and when it begins to migrate downwards.

2.0 Recommended Methodology for the HAB-BART Test Procedure

2.1 Standard Operating Procedure for Setting up a HAB-BART Tester

These are the standard steps in the setting up of a HAB-BART tester with a water-based sample:

1. Remove HAB-BART tester from the protective foam block.
2. Using a permanent marker, label the tester cap with the date and sample origin.
3. Unscrew the cap from tester. Set cap down directly onto a clean surface. To avoid contamination, do not invert cap.
   *Do not touch or contaminate the inside of the tester or cap during the filling procedure.
4. Fill the HAB-BART tester with the water-based sample to be tested until the fluid level reaches the fill line (15mls).
5. Place the cap back on tester and screw down tightly.
6. Place the tester **upside down on a flat surface for 30** seconds to allow time for the methylene blue crystals in the cap to dissolve into the waste water sample.
7. Place the tester in the palm of the hand; grip and using a wrist action rotate the testers **five times**. The ball will roll the full length of the tester each time and this will aerate the sample and evenly disperse the methylene blue (Redox indicator) into the sample.
8. After inversion, set tester upright and **immediately place** tester into a pod in the HAB-BART reader. The act of insertion automatically starts the test procedure and the letter "R" will appear on the lcd screen display on the front of the reader.
9. All testing should occur at 28 ± 0.5^{o}C as provided in the reader and away from direct sunlight. **Do not delay the start of the test once the tester is charged, it is recommended that all testers be set up one at a same time to assure accuracy. DELAY WILL CAUSE LOSSES IN PRECISION FOR THE TESTING.**
10. After testing is complete when the lcd display shows a number equivalent to the number of seconds from the start of the test. This is the time lag (TL) and can be used to determine the level of activity of the HAB in the water sample. If BART-READ (H4) is running in concert with the reader then the time lag is automatically converted to heterotrophic aerobic bacteria population given as predicted active cells / ml.
11. Safely dispose of HAB-BART testing vial using recognized regular biological disposal techniques including dedicated microwave or autoclave.

2.2 Procedure for Using the HAB-BART Reader

To set up a HAB-BART tester using the HAB-BART Reader, the following items are required:

1. One- 6 channel HAB-BART reader

2. Wall socket 120VAC

2.2.1 Installation of the HAB-BART Reader

1) Place reader on a horizontal, wood, metallic or plastic coated surface that is clean and dry.
2) Connect the power supply to the back of the HAB-BART reader.
3) Turn on power to the reader
4) Once power has been initiated, the HAB-BART reader will display a startup screen.
   Startup Screen: **Droycon Bioconcepts Inc.**
5) Plug reader in at least 60 minutes prior to beginning tests to allow time for the isothermic incubator block to get up to temperature (28±0.5^{o}C). The temperature of the block will be displayed on-screen with minimal and maximal values for the specific pod test..
6) Once the temperature is in range than the reader is now ready to begin testing.

2.2.1 Testing Procedure for the HAB-BART

1) Prepare HAB-BART tester following specific set procedures (see section 2.1).
2) FULLY INSERT HAB-BART tester in any one of the 6 channels available in the HAB-BART reader for each triplicate test. Ensure that the lower surface of the cap is flush with the top of the pod. This will automatically start the testing for that channel and the letter "R" will be displayed on screen for those channels.
3) HAB-BART reader should not be disturbed once testing has commenced.
4) When a positive detection of the reduction front moving into either one of the two light channels is recognized the test line on the screen will now read the TL (in seconds) determined for when the reduction front moved across the light pathway. To the left will be displayed the letter "U" (for up) and "D" (for down) to indicate the type of reaction detected.
5) The test effectively ends when there is a positive declaration of the time lag*.

* note that the time lag can be converted when running BART-READ (H4) to give a predicted population (pac/ml) as well as real-time graphing of the activity.

Cautionary Notes:

Basic concerns in the operation of this test method when using the HAB-BART tester include:

1) Make sure the HAB-BART tester is FULLY inserted into the reader to begin testing – the base of the cap should be flush with the top of the black pod channel.

2) DO NOT REMOVE HAB-BART TESTER WHILE TEST IS IN PROGRESS, this act can cause false positive detections to be displayed.
3) If the reader does not display properly when power is connected, disconnect power and re-apply after a few seconds. Remember that disconnecting the power will cause all channels to abort any test data stored in the reader.
4) In the event of a power outage the reader will continue to operate for at least 60 minutes but the isothermic block will not be heated (as an energy saving method) so that the block would cool down to a lower temperature. This would be recorded when the reader is connected to BART-READ (H4).

There are a number of restrictions as to where the HAB-BART tests can be carried out using the HAB-BART reader. These are summarized as:

a) The readers are for **Indoor Use only** but can be set up in a mobile laboratory or field station provided that the area is at room temperature (23±1^{o}C) or cooler (down to 8^{o}C). Note that the reader will continue to perform effectively in a vehicle or vessel that is in motion and so it can be used on ships, in road/rail vehicles and in planes or dirigibles.
b) Keep HAB-BART reader and HAB-BART testers out of direct sunlight.
c) Reader must be placed on a level, horizontal surface that is free from static charge. The surfaces should therefore be of a metal, wood or plastic construction or plastic coated.
d) Reader should not be disturbed while testing is in progress.
e) Reader should be kept away from any risks of water/liquid spills..

2.3 Disposal of the Finished HAB-BART Testers

Hygienic and safe disposal of the completed HAB-BART testers is an essential part of the protocol. This is dictated by the fact that there could have been a growth of bacteria in the test media and that this medium may also contain some potentially pathogenic bacteria. Disposal of completed HAB-BART testers should therefore take place following strict microbiological guidelines to ensure hygienic disposal. These include the disposal of the spent HAB-BART packed loosely into a standard double thick, 4 mil polypropylene biohazard, and autoclavable bag. It is recommended that the staff responsible for filling the bags should wear protective gloves while ensuring that all of the completed HAB-BART testers are tightly sealed before being placed in the bag. Once the autoclave bag has been filled then it should be sealed following the manufacturer specifications and placed within the steam-operated sterilizer (autoclave). To sterilize the spent tests the sterilizer has to apply a minimal steam pressure of 15psi to achieve an internal temperature of at least 121^{o}C. Commonly for sterilization there is an increase in the time from 20 minutes to between 40-45 minutes with minimum pressures of 15 psi and temperatures of 121^{0}C. Once the bag of spent HAB-BART tests have been sterilization then the sealed bag can now be disposed of with the normal garbage.

Appendix III, Specifications, HAB-BART system

HAB-BART tester

Per unit:	Weight:	15.71 g
	Height:	90 mm
	Diameter (with cap):	27 mm
Packaging:		
	Number per box:	15 testers in 3 rows of 5 in a foam protector
	Vial construction:	Injected medical grade polystyrene
	Cap construction:	Injected food grade polypropylene
	Foam protector:	90 mm x 72 mm x 335 mm (w, h, d)
	MSDS sheet:	One per carton
	Packing rate:	Five boxes per cubic foot of carton space
	QM certificate:	One per box of 15 testers
	Moisture barrier:	Aluminum foil (preformed and sealed)
	Shelf life:	Four years
	Storage conditions:	Cool, dry place

Tolerances:

Storage temperature:	2 to 42^{o}C
Salinity tolerance:	Maximum 4% sodium chloride equivalence before Methylene blue color shifts towards green
Solvents:	Gasoline, acetone – destructive Diesel – 5 day tolerance limit
pH operating range:	1 to 12.5

HAB-BART reader

Per unit:

Weight:	5.5 kg
Dimensions:	220 mm x 145 mm x 270 mm (ht.wd.dp)
Outer case:	16 gauge (3 mm) aluminum
Finish:	Black matt powder coated
Circuit boards:	3 major, 12 minor
Back up battery:	minimum 60 minutes operating
Voltage:	110 volts AC
Power up switch:	Rear side of reader
Amperage:	<2 amps draw
Display:	Back-lit liquid crystal display (4 lines)
Warranty:	One year replacement, extendable
Static protection:	Rubber feet and three grounding points
Communication:	DB9 male port.
Repair:	Return to manufacturer
Monitoring scale:	1 to 255 sorption units (s.u.)
Critical O_2 concentration:	0.056ppm O_2

BART-READ (H4) software

Language:	Visual Basic / C++
Size:	3.4 KB compressed
Available:	One CD per reader
Up-dates:	Available at web site www.dbi.sk

Problem resolution

Hardware:	Electronics dept.(306) 585 1762
Software:	Computer dept. (306) 585 1762
Technical:	Research & development dept. (306) 585 1868
E. mail enquiries:	Sales@dbi.ca

Manufacturer information

Address:	315 Dewdney Avenue
City:	Regina
Province:	Saskatchewan
Country:	Canada
	S4N 0E7
Telephone:	(306) 585 1762
Fax:	(306) 585 3000
Web:	www.dbi.ca
E-mail:	sales@dbi.ca
ISO 9001:2000	Certified, September, 2001 (BDC)
	Re-certified, October, 2002 (BDC)
	August, 2003 (BSMI)
ETV (Canada):	IRB-BART tester, February, 2002
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