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Protocol

DBHSOP06

HAB-BART

Testing Protocols for Heterotrophic Bacteria

HAB-BART tester involves two reaction types relating to the locations where the HAB have been growing. The tester uses a selective medium that encourages the growth of the two major types of HAB. This document provides a structured and logical protocol to allow standard laboratory QM procedures to be adopted. There are two parts to the protocol: (1) Interpretation of accepted and known reaction patterns generated by HAB; and (2) Establishment of a link between the time lag (time interval between the start of the test and the first observation of a positive reaction) and a semi-quantitative prediction active HAB in the sample under test.

1. Definition of Heterotrophic Bacteria

Heterotrophic bacteria are that group of bacteria that are able degrade organic material in the environment being sampled. There are two types of degradation that can occur. One functional group degrades organics oxidatively with the use of oxygen through a respiratory function (aerobic degradation) The second functional group degrades the organics reductively with the releases of acidic and gaseous products but the organics are not broken down completely (anaerobic degradation. These two groups (aerobic and anaerobic degraders can be defined as heterotrophic bacteria using the definition below:

> All bacteria that have the ability to degrade organic materials are considered to belong to the heterotrophic bacteria whether that degradation involves respiration (aerobic) or fermentation (anaerobic). Daughter products from these activities will be carbon dioxide and biomass (aerobically) or a mixtures of gases with smaller organic molecules some of which may be acidic (anaerobically).

It should be noted that many of the heterotrophic bacteria can function both aerobically and anaerobically. These bacteria are known as facultatively anaerobes and are very common. “HAB” as the name for these bacteria recognizes this through defining the group as “heterotrophic aerobic bacteria” since the bulk of heterotrophic bacterial species are capable of aerobic functioning in a suitable environment.

In the HAB-BART test, the reactions fall into two prime categories relating to the function of the bacteria in the sample. The primary mechanism for determining these groups is the oxidation-reduction potential (ORP) in the sample. This shift is determined by the application of methylene blue in the tester that turns blue under oxidative conditions and clear under reductive. The manner in which the methylene blue shifts from a blue to a clear color indicates which of the two major groups of heterotrophic bacteria is dominant. This si differentiated below:

UP reaction – aerobic function

Aerobic bacteria generally remove the oxygen first from the base of the tester since reduction first begins at the bottom of the HAB-BART tester. As the available oxygen diffusing downwards through the culturing fluids is outstripped by the bacterial demand for oxygen then so the reductive front moves up the tester changing the color of the fluids from blue to colorless or a light shade of yellow depending upon the type of bacterial activity. Since the reductive (bleaching) zone moves from the bottom up through the fluids this is known as an “UP” reaction.

DO reaction- anaerobic function

Anaerobic heterotrophic bacteria work in the reverse manner to the aerobic bacteria. Here the bacteria attempt to block the diffusive movement of oxygen down into the fluids around the ball. This blocking action is done to allow the anaerobic bacteria that are sensitive to oxygen, a place to grow in the absence of oxygen. When anaerobic bacteria dominate in the fluids these activities causes biocolloidal clouds to form below the ball. This restricts the movement of oxygen downwards. This activity can be witnessed by pale blue patches appearing and moving in that zone as the methylene blue shifts partially to a reductive state. After this phase of partial mobile reductive events then all if the oxygen is removed first from a position about 5 to 7mm below the ball causing a major reductive event that now decolorizes (reduces) the methylene blue. This reaction therefore proceeds from the top downwards and so it is referred to as a down (DO) reaction.

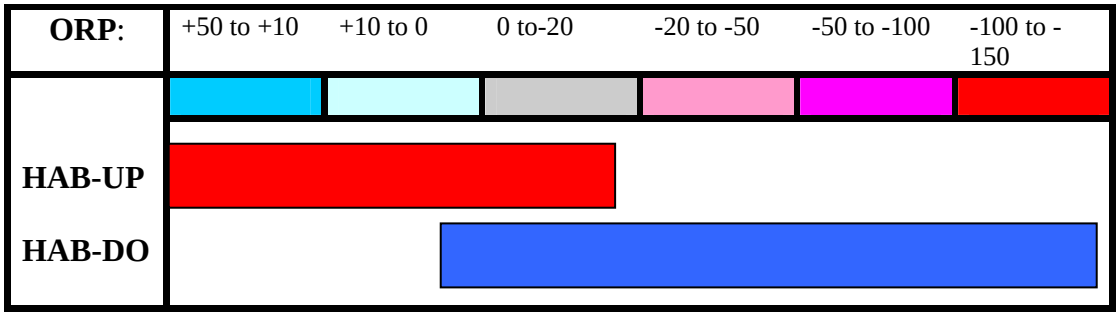
These are the only two recognized reactions (UP and DO) that are of major importance in the identification of the heterotrophic bacteria. For the HAB-BART tester, this bleaching effect (reduction of methylene blue to a colorless form) is the prime diagnostic feature with the time lag to the occurrence of the event correlating to the population size and activity levels in the sample.

While some heterotrophs generate UP reactions and other down reactions, in both case the HAB-BART tester will gradually become bleached (i.e. devoid of the blue color) except around the ball where 1 to 3mm of the oxidized blue-form of methylene blue remains. This blue ring is supported by the copious amount of headspace oxygen that prevents this region of the ball from becoming reductive. The rate at which the bleaching of the methylene blue occurs either UP or DO can be used to determine the ORP of the original sample by comparing the rates at which the bleach zone moves up or down using the HAB-BART reader to monitor the event routinely and BART-READ software to record the data in real time and generate the graphs and interpretation of the ORP value.

2. Recognizing the Reaction Patterns

These observations can be summarized in the following ORP gradient chart (Figure one):

Figure One, Relationship of HAB-BART reaction to ORP

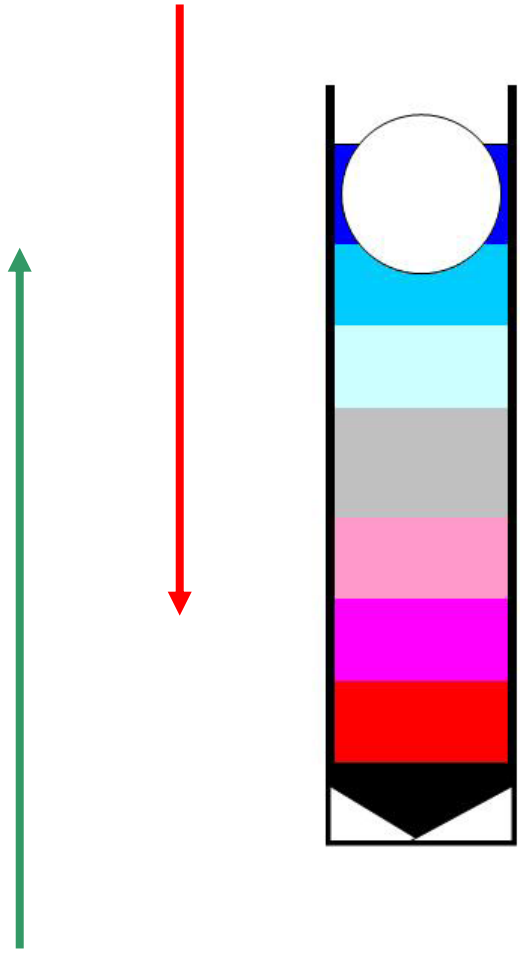


Note: HAB-UP refers to an HAB generating an initial decolorization (bleaching) of the methylene blue in the base of the tester; HAB-DO refers to an HAB that produces the initial bleaching just beneath the ball which then spreads downwards. In both cases there remains a ring of blue just the equator of the ball. Here the likely occurrences of HAB-UP or HAB-DO in relation to the ORP gradient from >+50 mV (blue) to < -150 (red).In the operation of an HAB-BART tester, it is normal for an ORP gradient to form if there is any microbial activity occurring within the HABBART tester. In the HAB-BART test, this generation of the ORP gradient causes a focussing of the HAB bacterial community's activities in relationship to the types of bacteria that are present and active.

In the HAB-BART tester, there are two primary functions:
1. The oxygen from the headspace in the tester diffuses down passed the ball. (red arrow down
2. Selective chemicals diffuse up from the base (green arrow up).

Figure Two, Functionality of the HAB-BART tester

OXYGEN DIFFUSES DOWNWARDS – OXIDATIVE



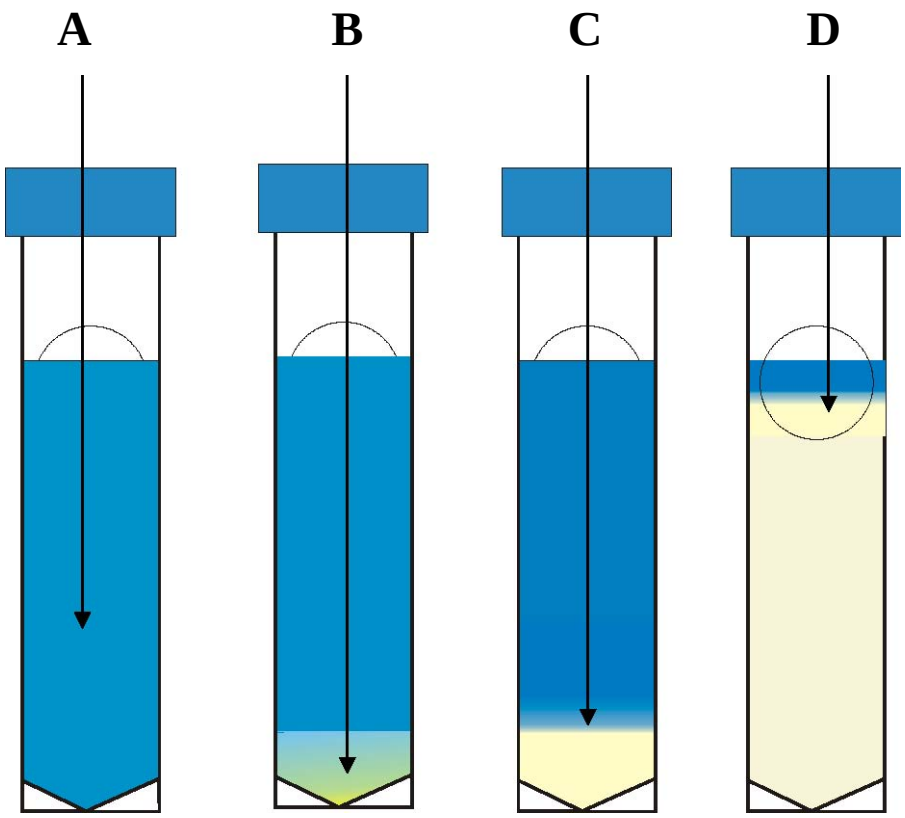
NUTRIENTS DIFFUSE UPWARDS – REDUCTIVE

Note that the colors for the ORP are the same as rendered in the figure one above. Directions of the bleaching event are shown to the left of the figure with the DO reaction direction shown in red and the UP reaction shown in green.

3. Recognizing the Non-HAB Reactions of Possible Significance

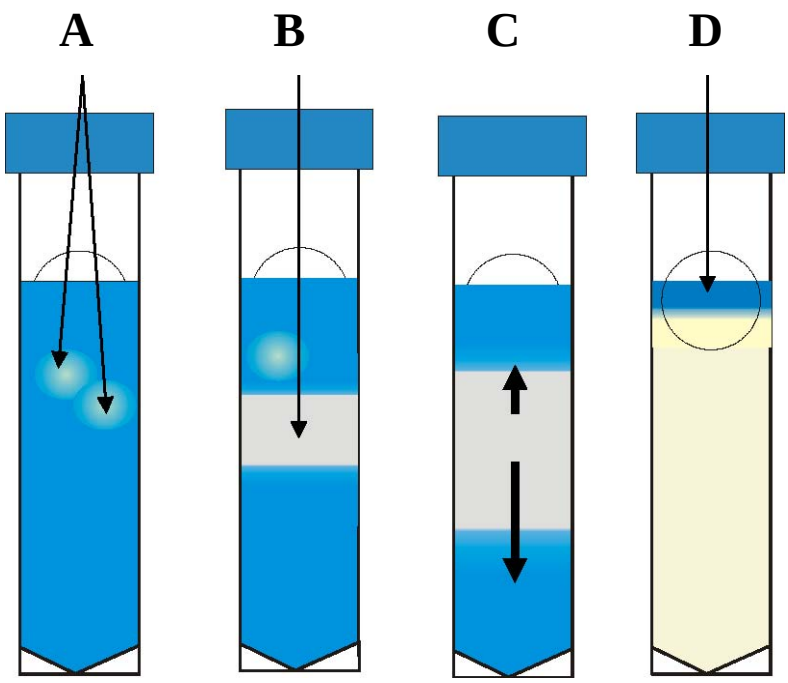
There are two reactions that can commonly occur in the HAB-BART tester. These are described below as Figures three (UP) and four (DO).

Figure Three, Illustration of the UP Reaction



The UP reaction in a HAB-BART tester (A) begins with a decolorization in the base of the tester (B) that commonly has a yellow tinge to it and is the result of the medium pellet dissolving into the culturing fluid. A positive is recognized (C) when the decolorization (bleaching) begins to move up the side of the tester leaving a pale yellow or clear zone trailing below. This movement of the bleaching up the side of the tester indicates that the test is positive. Following the declaration of a positive, the bleach (reductive) front continues to move up the tester and stops 1 to 3 mm beneath the surface.

Figure Four, Illustration of the DO reaction

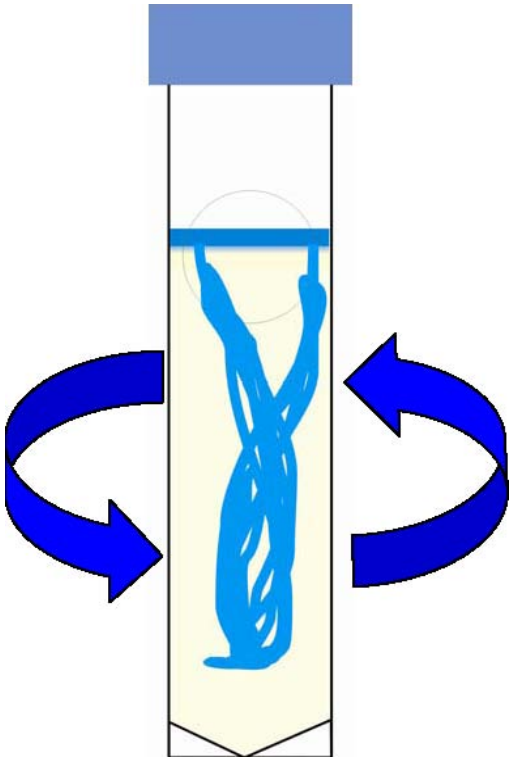


When a DO reaction occurs, the first signals are some clouds forming just below the ball (A). These clouds cause a partial bleaching (reduction) but are very mobile and so cannot be considered an indication of a positive reaction. A positive DO reaction is recognized when a lateral bleach zone forms below the ball (B) and the time lag is based on this event. This lateral bleach zone expands (C) generally faster downwards than upwards until the bleaching extends from the bottom of the tester to just below the surface (D).

While these two reactions are the only two recognized for the identification of aerobic (UP) and anaerobic (DO) heterotrophic bacteria, there is one other common event that occurs with UP reactions when there is a large and very active aerobic population. This reaction is summarized in Figure five Here there is a secondary reaction that can occur once the UP reaction has been identified. What happens at this point is that the bacteria begin to generate slime columns that are structured from the edge of the reductive (bleached) zone upwards to around the ball. These structures remain blue (oxidized) even after the background culturing fluids has turned reductive. What is seen at this time are these mobile structures moving vertically and connecting from around the ball to the reductive zone below. It appears that these slime structures bring additional oxygen down from the headspace via the liquid space around the ball. This phenomenon is known as the “twister” effect since the time lapse photography of such HAB-BART testers show

these blue vertical slime columns twisting around each other. Over time these twisters weaken and eventually collapse. When employing the BART-READ software program to monitor the twisters then the real time graphs develop a “dog tooth” effect as the upper light channel changes in sorption as the structures move in and out of the light beam.

Figure Five, Observation of a Twister in an UP reaction.



When a twister forms it retains the blue color of the oxidized methylene blue even when the background has bleached (reduced). Time lapse photography shows that there is a rotation of the structure of about two to three rotations per hour. This is detected using BART-READ as a “dog toothed” form of graph as the sorption detected on the upper light channel changes dramatically (Figure six). While the twister is a scientifically interesting event it is not directly used in the projection of the population which is dependent upon the first time lag (commonly generated in seconds). If this time lag is first generated by the lower channel then an aerobic function (UP) is declared. If it is the upper channel that first declares then that is an anaerobic (DO) reaction. The relationship of the two time lags is used in BARTREAD to project the likely ORP value for the original sample. This can be found when going to the large format figure function and observing the graphing and interpretation function. Occurrences of the twisters is viewed as an explanation for the erratic data generated in the upper channel (blue) after a positive time lag has been declared for an UP reaction. Twisters are rarely formed during DO

reactions which would indicate that the twister is a product of extremely active aerobic bacterial populations.

Figure Six, Impact of Twister in an UP reaction on Real Time Graphing

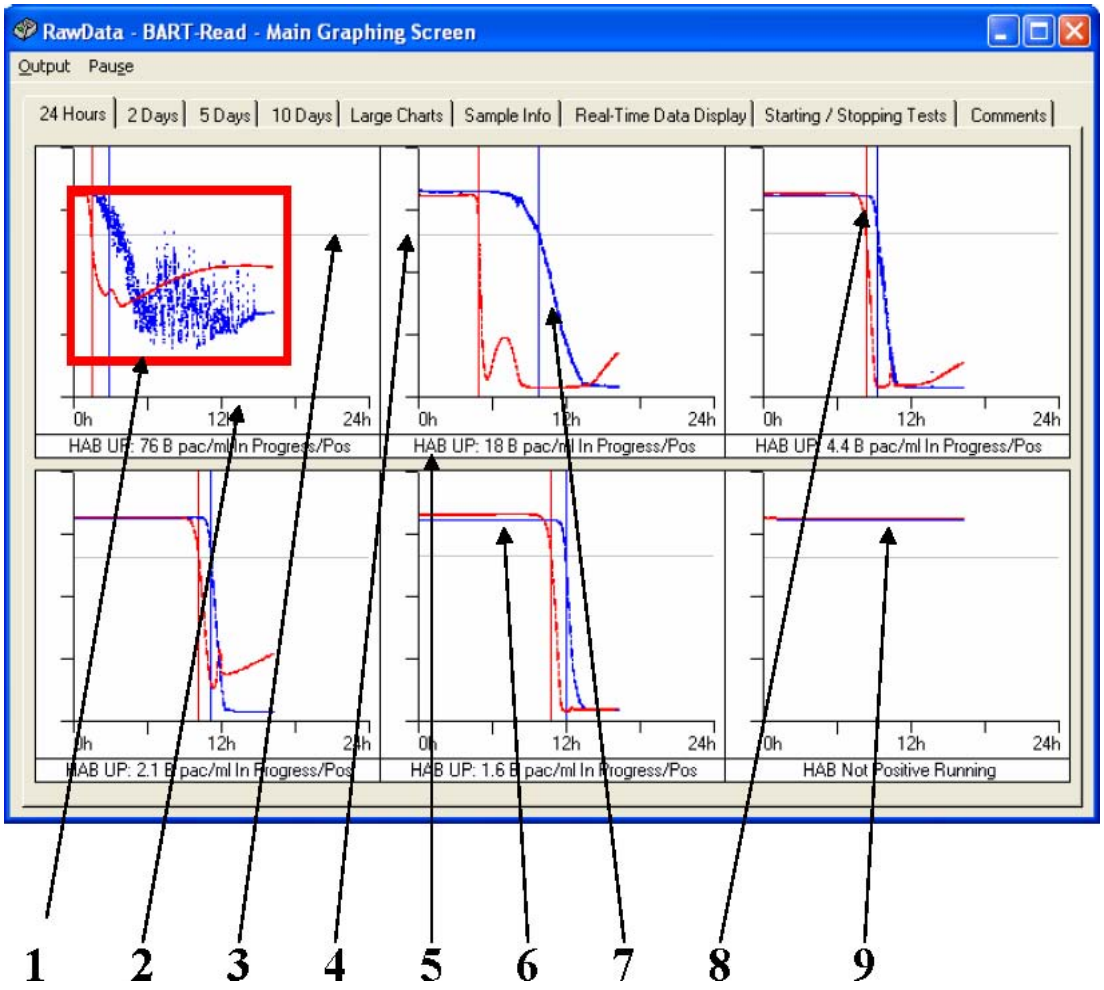


Figure six shows six real time graphs being performed in two rows of three. The upper left graph demonstrates a typical twister effect (1, red box, blue upper channel). X axis shows the time in hours of the test (2) with the decision called when the first line crosses the threshold line (3). Y axis shows the relative sorption value (4) over a relative range of sorption with high sorption at the top of the axis. Interpretations are shown (5) beneath the individual graphs and give the interpreted values along with the time lag. Commonly the lines remain fairly stable (6) prior to the reductive (bleaching) process and then decline rapidly (7) if there are no twisters. Decisions are declared by vertical red (lower) and blue (upper) lines created (8) at the moment that the decision is made. A control graph (9) is included to indicate a negative detection with flat lines for bopth of the light channels.

4. Qualitative Interpretation of Reaction Patterns

There are two reaction patterns relevant to the HAB-BART which is UP and DO. These two reactions signify that different communities of bacteria (see Figure one) are dominating based upon the aerobic and/or anaerobic abilities to degrade organics.

Table One, Reaction Patterns in the HAB-BART tester

Reaction	Reaction pattern	Diagnostic comments
UP	Reaction forms in the base of the tester as a bleached zone that might have a yellow color against the background of blue	Heterotrophic bacteria are fundamental able to be more active under an aerobic environment. This does not mean that these bacteria are only capable of aerobic functions, some can also function anaerobically.
DO	Test generally shows some bleaching that is transitory just below the ball. This is replaced with a lateral zone of bleaching that spreads downwards faster than it ascends. .	Anaerobic heterotrophic function dominates to suggest that the heterotrophic activity is primarily fermentative with the degradation to acids such as the fatty acids and a range of gases including nitrogen, carbon dioxide, hydrogen and methane. Some of these bacteria may be capable of aerobic functioning but they are active anaerobically.

Diagnostic interpretations are based on the type of reaction observed. For UP reactions this will mean that the HAB are active at sites that are oxidative or mildly reductive (at the redox front see also Figure two above). Here the HAB are forming a part of bacterial consortium dominated by aerobic bacteria with the HAB dominating the biofilms. For a DO reaction, this would mean that the HAB are growing under relatively reductive conditions and will be associating with other anaerobic bacterial consortia. In general the DO types of HAB have proved to be more difficult to control in a natural environment than the UP since it is generally more challenging to treat reductive zones that are often deeper in porous media and away from the influences of oxygen.

The term “Heterotrophic bacteria” implies strongly that all of the microbes cultured using the HAB-BART tester are most likely to be involved in the degradation of organics. To achieve this, the HAB-BART tester employs a nutrient pellet dispensed into the base of the tester. Chemicals released from this pellets than encourages the activities of the HAB in the sample being tested. Some of the chemical ingredients used in the pellet are listed in Table two.

Table Two, Major Significant Chemicals employed in the HAB-BART tester

Chemical	Function
Peptones	Rich Source of amino acids that stimulates microbial cell reproduction to maximize cell activity
Phosphate	Rich source of phosphorus to maximize cellular activity with a high ability to generate energy intensive activities.
Methylene Blue	Methylene blue is used as an indicator of the ORP status in the HAB-BART with the chemical going to a blue color in the event of an oxidative condition and a clear color when these conditions becoming locally reductive.
Polymerized carbohydrates	Rich source of organic carbon that stimulates the bacteria to utilizes these chemicals for growth and energy production.

5. Quantitative Interpretation of Time Lags generated by HAB-BART testers

Population counts have been traditionally set by such determinations as the number of countable colonies on a known agar surface that have grown from a know volume of sample. This is commonly referred to as the number of "colony forming units" (c.f.u.) and is in common use in microbiology laboratories for reporting populations. For the BART methodologies it is not possible to count colonies since a liquid culture is employed for the cultivation of the microorganisms. Colonies therefore cannot be seen and certainly cannot be counted and so an alternative approach has been employed. As a basic premise it is assumed that the bacterial population in the sample is formed by two primary parts: (1) active cells that have the potential to be cultured in the HAB-BART tester; and (2) active and inactive cells that do not have such abilities to be cultured under the conditions established in the tester. In the evaluation of population size for the BART testers, the primary method is to take the link between the speed with which the first reaction happens, the activity level of the cells in the sample and then project a population. In the use of speed to determine the bacterial activity, it is the time lag (commonly measured in seconds, hours or days) that is used to predict the population size. Here the preferred unit for quantifying the population is predicted active cells (p.a.c.). This means that the unit of expression (i.e. p.a.c.) relates to an evaluation of active cells in the sample and not the total number since a fraction of the cells would be either dormant or unable to react to the conditions established in the HAB-BART and generate one of the two distinguishable reactions. The population predictions (based commonly based on either the UP or DO time lags is given in the Table three. Here tow temperatures are shown for the projection of populations (room temperature and 28°C that is considered optimal for the application of the HAB-BART reader. Populations are

based on the first time lag generated when the reader is used.

Table Three, Predictions of HAB Populations using time lag

Time Lag			HAB population in p.a.c./ml			
seconds	**hours**	**days**	**12°C**	**22°C**	**28°C**	**36°C**
21,600	6		350T	8.1B	76M	230M
43,200	12		300T	1.5B	33M	73M
64,800	18		250T	320M	15M	25M
86,400	24	1**	210T	77M	6.9M	9.3M
172,800	48	2	88T	650T	450T	290T
259,200	72	3	32T	19T	48T	20T
345,600*	96	4	10T	1.4T	7.4T	2.3T
	120	5	2.7T	210	1.6T	440
	136	6	610	52	450	120
	168	7	120	18	160	42
	192	8	19	8	65	18
	216	9	2	4	31	10
	240	10	1	3	17	6

Note: B – billions; M – millions; T – thousands; units are in heterotrophic bacterial cells as p.a.c. / ml (which are equivalent to colony forming units or cfu/ml). The program BART QuickPop is available on the web site (www.dbi.ca) where more precision is desired. For the purposes of uniformity the time lag to the first recognized UP or DO reaction is used to determine the population. In the calculation of the population as predicted active cells per millilitre (p.a.c. / ml) the data is automatically modified to correct from the 15ml sample size to predict the p.a.c. / mL in the HAB-BART tester.

6. Establishment of Standard Protocols.

A standard protocol is limited to specific conditions in the manner and from with which the sample will be received for testing, the nature of the set up of the test procedure relevant to the sample, conditions for the ongoing observation of the HAB-BART during the testing phase, and the manner in which the time lag and reaction patterns will be observed. There will be three protocols listed below: (1) water and waste water samples; and (2) semi-solid and solid samples such as sludges and soils (3) oils.

Standard Protocol for setting up a HAB-BART tester

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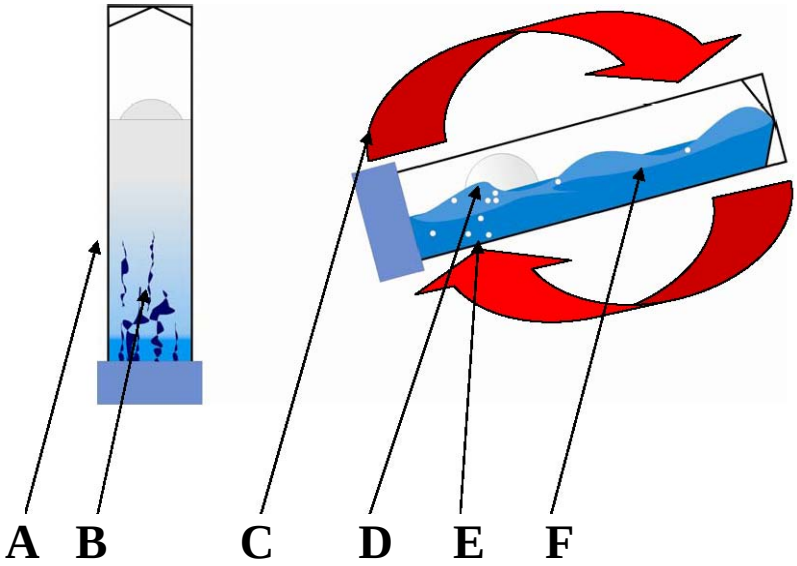
Regardless of whether a water, oil or soil sample is employed there is a standard operating procedure for charging the tester with the methylene blue. Because methylene blue does not have an adequate storage capability in the liquid form or as a crystallized material associated with other chemicals, it is dried in the inner cap of the tester. Here the methylene blue maintains specifications for at least four years when stored in the standard foil pouches. To set up the HAB-BART tester this methylene blue has to be dissolved into the liquid (i.e. water sample or sterile phosphate buffer) and then shaken to aerate the liquids to allow saturation with oxygen from the headspace gases. It is very important to ensure oxygen saturation since this ensures a highly oxidative condition for the start of the test. The standard procedure is summarized in Figure seven. There are two stages in this activity. First the charged tester is placed upside down for thirty seconds so that the dried methylene blue in the cap dissolves and diffuses into the water. Second, the tube is shaken by a rotating wrist action five times which causes the ball to travel the full length of the tube ten times causing the air from the headspace atmosphere to be dispersed into the liquid to reach saturation. This agitation also disperses the microorganisms throughout the liquid.

Figure Seven, Preparing a HAB-BART tester for a test



This figure shows the manner in which the methylene is dispersed into a HAB-BART tester. First the tester is placed upside down for 30 seconds (A). Here the methylene blue dried in the cap diffuses into the liquid commonly as a series of dark blue columns (2). After 30 seconds of standing upside down the tester is now rotated (C) by a vigorous wrist action five times causing the ball (D) to move from one end to the other end of the

tester ten times. This causes the air to get mixed into the liquid (E) so that the liquid becomes saturated with oxygen and the liquid develops an even blue color. The tester is now ready for incubation with periodic observations for significant activities. .

Recommended Protocol for Water, Condensate and Waste Water Samples
Cautionary conditions relating to water sample collection and storage

There are standard procedures for the collection of water and waste water samples but these are not so well defined for other waters. Essentially Heterotrophic bacteria tend to be able to survive 2 or 3 days in water samples with no significant loss in activity if the samples are kept over ice or in a refrigerator at $<8^{\circ}C$. There are a number of basic considerations to ensure precision and validity in the final interpreted data. These can be summarized as needing to include the following conditions:

1. If the sample is going to be tested within four hours of collection then the sample may be allowed to acclimatize to room temperature at a site away from direct sunlight in a place where there are no vibrations or disturbances of any type.
2. If a water sample is taken from a continuous stream flow then the sample should be taken at midpoint as far as possible to avoid contamination from any growth occurring on the walls of the conduit.
3. If it is desired to examine the types of heterotrophic bacteria growing attached within the conduit with which the water is in contact, then changing the environmental conditions through stopping or radically changing the flow rate will cause stress amongst the attached bacteria including the HAB. There is likely to be more releases of HAB into the water.
4. If a water sample has to be collected longer than four hours prior to testing then the sample should be cooled down over ice to approximately $4^{\circ}C$. Water samples for HAB determinations can be kept for 72 hours in this state with no significant changes in activity.
5. Water samples should always be kept in sterile containers whenever possible to reduce the risk of contamination.
6. In the event that sterile containers are not available in the field for on-site testing then the outer vial of the HAB-BART field tester may be used to collect the sample.
7. It is not necessary for the sample container to have a chemical tablet to neutralize residual chlorine since: (a) the heterotrophic bacteria do have a resistance to the normal levels of residual chlorine in water; and (b) the HAB-BART incorporates a chlorine neutralizing chemical to eliminate any potential inhibition.
8. It is not necessary to completely fill the sterile sample container with the sample water since this would cause some restriction in the available oxygen since it would be used up through indigenous respiration leading the a more reductive ORP which would reduce possible overgrowth by aerobic bacteria and reduce the stress on the oxygen sensitive heterotrophic anaerobes .
9. Cleanliness should be imposed in all stages of the collection and handling of the sample and rigorous efforts need to be applied to assure no casual contamination of the sample in any manner.
10. It is recommended that latex gloves be worn by the operator when working with

the HAB-BART testers.

There are two different types of HAB-BART tester are the: (1) field tester; and (2) laboratory tester. The field tester is designed for use away from the laboratory and can be charged with the sample close to the sampling site and monitored in the field at a suitable temperature. This type of tester includes an outer vial which surrounds and protects the inner vial in which the test is actually performed. The other type of HAB-BART tester is meant to be used in the laboratory under normal conditions for the handling of microbiological materials. This tester includes the essential inner tester necessary for undertaking the HAB-BART test but without the use of the outer vial. The field tester therefore provides additional protection for the user from potential leakages of odorous gases or cultured fluids that may occur from the inner tester.

Protocol DBHFWO6
Using the HAB-BART (Field version) tester to test for HAB in water

Filling the HAB-BART tester involves a sequenced approach that ensures that there is no casual contamination of the tester and that a suitable volume of sample has been added. Generally when the field HAB-BART is going to be used, it would be used in a field setting (rather than laboratory) and will be read visually on a daily basis as far as is conveniently possible. Where greater precision is desirable then it is recommended that the laboratory tester be employed along with the HAB-BART reader systems Instructions for the charging the HAB-BART field tester is described below:

The field tester comes in packs of three each separately sealed within an aluminum foil pouch. To remove a field tester from the pouch, locate the “tear down” tab on one end of the pouch, grip it tightly and pull it down over the pouch. This will exposed the field tester that can then be removed. Make sure that there is a clean flat surface to place the tester in a place where it is not likely to be knocked over.

The container containing the sample to be tested should be placed next to the field tester on a clean surface and it is important to make sure that there is not likely to be any strong winds or air currents that could bring dust into the area where the test is going to be set up. It is recommended that latex gloves be worn during the setting up of the HAB-BART testers to reduce the risk of contamination. Follow the sequence of activities as described below:

(i) Label the top of the cap on the outer vial of the field tester with the essential information to recognize the sample (e.g. name, date, time) using a black fine point permanent marker. Do not write on the plastic walls since this might seriously impair the ability of the operator to recognize positive reactions. Make sure that the ink from the marker is dry before attempting to charge the field tester. If this is not done then sample information may become smudged and unreadable.

(ii) When ready to charge the field tester with the sample, the first step is to unscrew and remove the outer cap of the tester. It should be noted that the act lifting up the outer cap causes the inner tester vial to be lifted up as well.

(iii) As the outer cap is lifted up clear of the outer vial then gently remove the inner tester from the outer cap and place that cap down on a clean surface without turning it over and place the inner tester next to the sample to be tested. It is strongly recommended that charging of the tester be done one at a time to reduce the potential for inadvertent mixing of the samples and testers.

(iv) Unscrew the inner cap from the inner tester and place the cap down on a clean surface without turning it over. Note this cap is not labelled with the sample information and so it is important to remember to do one test at a time to prevent mixing up of samples. Note also that the contents of the inner HAB-BART tester are now exposed to possible contamination from the outside environment and so the next steps should be done quickly. Unscrew the cap from the sample container and slowly pour sufficient water into the inner tester vial to bring the water level up to the fill line indicating 15ml of sample has been added. The ball will float up on the rising water column and the water level is observed to be at the fill line and against the ball. When pouring water into the inner tester vial, every effort should be made to direct a stream of the sample water over the center of the ball rather than allow it to trickle down the side. Care should be taken to ensure that the final water sample level is to within 2mm of the etched fill line on the inner vial of the BART tester. For the effective use of the field tester, maximum tolerance for error for filling the HAB-BART tester is 5% so that the amount of water sample being tested falls within the range of 14.25 and 15.75ml. It should be noted that the sample container retains a headspace of air over the sample and so some oxygen will diffuse down into the liquid sample. To assure that there is oxygen in the sample along with a dispersion of any settled or particulate materials within the sample it is recommended that the sample container be inverted five times before being used to charge the field tester. This assures a better level of precision since the oxygen concentration at the start of the test would be at a saturated level and the possible bacteria of interest are more likely to be evenly dispersed throughout the sample.

(v) Once the inner tester vial has been charged, immediately screw back the inner cap down on the vial. This cap does not need to be screwed down hard. Do not shake or disturb the contents of the inner tester vial in any manner. Lift the outer cap without turning it over and clip the inner tester vial back into the underside of the outer cap. Now grip the outer cap (inner charged tester attached) should now be tightly held inside this cap). Lower the inner tester back down into the outer tester vial. Screw the outer cap down firmly but without excessive force. The charged field tester may now be moved to a safe place for periodic observation. In moving the charged tester remember that the test has started and that complex diffusion fronts for oxygen (moving downwards) and nutrients (moving upwards) are occurring and that severe movement may affect the precision of the tester.

(vi)Testing begins as soon as the sample has been added. If the sample has a much lower temperature than the temperature at which the tester will be maintained (e.g. room temperature) then there is a probability that excess oxygen might be released into the culturing tester causing gas bubbles to form on the walls. This is not a positive reaction. To minimise this risk, the sample should be given time acclimatise to the temperature at which the testers will be monitored (e.g. room

temperature). This acclimatization would be dependent upon the sample bulk volume but should, under normal conditions, take no longer than one hour.

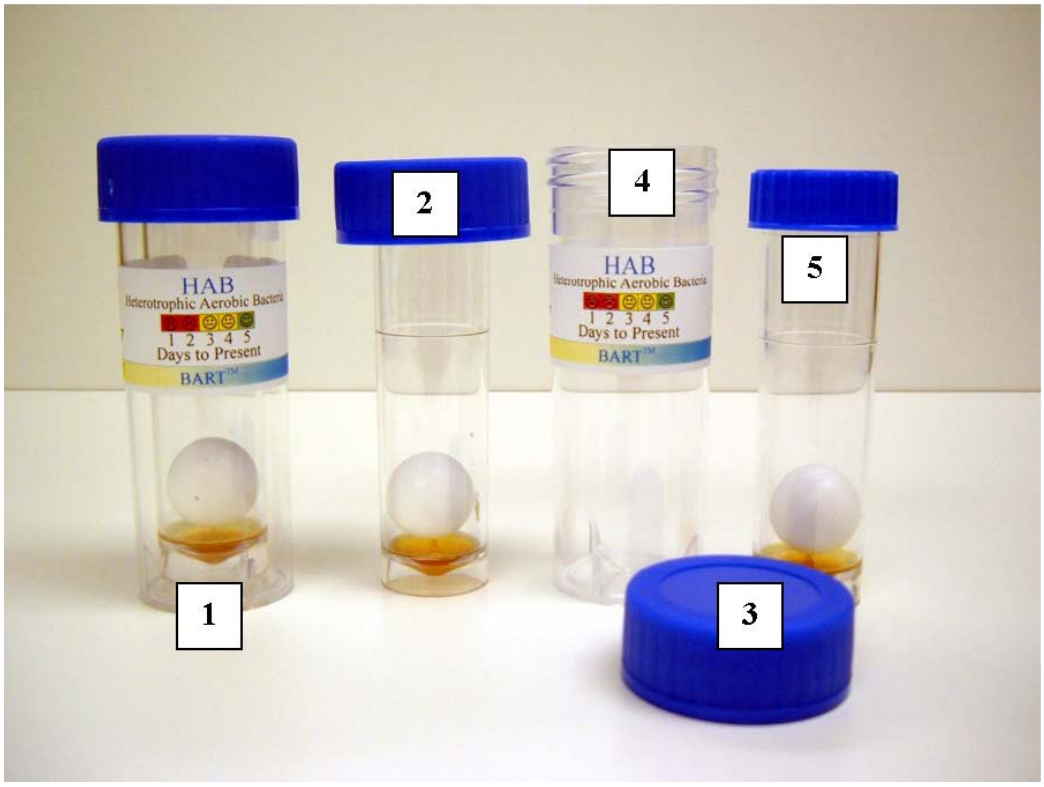


Plate One, HAB-BART Field Tester. The sequence for preparing the HAB-BART field tester for testing is summarised as: (1) take a field HAB-BART tester; (2) unscrew the cap and place on clean dry surface; (3) remove the inner tester vial from the cap; (4) put the outer vial aside until required at the end of charging the tester with the sample; and (5) fill the inner tester vial with the 15mL sample (to the fill line). Note these activities are undertaken in rapid succession leaving the operator with items 2, 4 and 5. The next plate shows the method for charging the field tester with sample.

(vii) Incubation temperature commonly recommended for the field HAB-BART tester is room temperature which is considered ideally to be between 21 and 25 ^{o}C away from direct sunlight that could cause internal heating of the tester. If the temperature goes above 25 ^{o}C then there is most likely to be an increase in the rate of iron related bacterial activity and the time lags for given population sizes can be expected to drop. This would cause higher populations of HAB to be predicted. If the temperature drops below 21 ^{o}C then this may commonly slow down the level of bacterial activity causing losses in precision (where duplicate tests are performed) and much lower predicted populations.

(viii) Observation for the occurrence of the UP or DO reactions in the tester should ideally be performed daily until day 6 and then again on days 8, and 10 if no reactions are observed. Critical days for the observation of activities and reactions are days 1, 2 and 3 along with such other days up to day 10 if the objective is to obtain a qualitative

and/or quantitative assessment from the sample in the tester. Remember that the only significant reactions are the UP and DO reactions and that only one of these reactions can occur; There are no subsequent significant reactions for the HAB-BART tester.

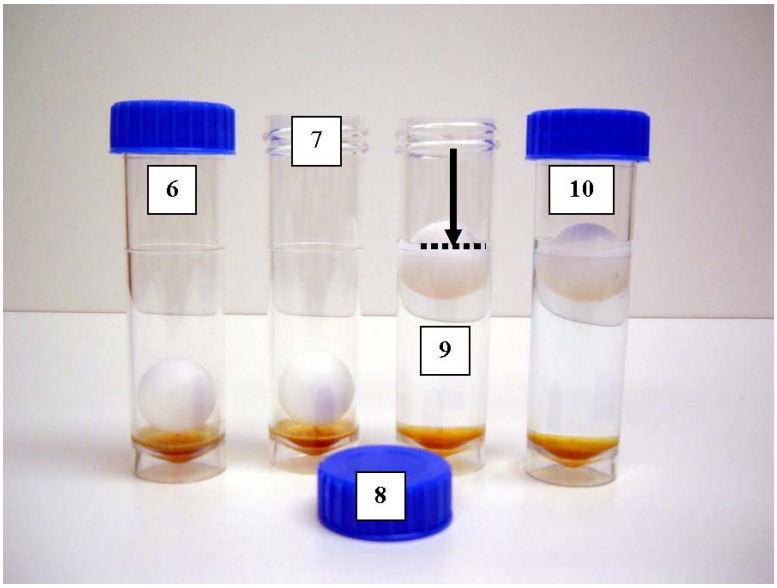


Plate Two, Filling the Inner Vial of the HAB-BART Field Tester In the above plate the sequence for charging an inner HAB-BART tester (6) as requiring the following steps: (7) unscrew the cap; (8) place the cap down on a clean surface without turning the cap over; (9) carefully fill the tester vial with the sample until the water level is at the same level as the fill line, note that the ball floats up as the water level rises; and (10) screw the inner cap back onto the vial without using force. Once this has been done then the outer cap can be positioned over, and pressed down onto, the inner test vial that will now become seated into the outer cap. Once this has been done then the outer cap (with the inner tester attached can be returned to the outer vial and screwed down. The test for the presence of iron related bacteria has now started.

(ix)To observe a tester for significant reactions then it is important to follow the same procedure each day (and at the same time of day as the test was started). This procedure would consist of lifting the tester up towards a light such as a fluorescent light fixture or the northern sky during daylight hours. The ideal position to view the tester is to place it directly in front of the light source at arms length. The culturing tester would begin by having a blue color which would, when there is a positive, move to a clear, clouded or pale yellow color. See Figures four and five for the reactions patterns typical of a UP or DO reaction.

(x) Finished tests should be disposed of using one of the techniques recommended in below.

Protocol DBHLWO6

Using the HAB-BART (laboratory) tester to test for HAB in water

It is strongly recommended that the HAB-BART reader be employed when undertaking procedure DBHLWO5 to obtain satisfactory precision. Protocols for the reader are covered in BART-READ for HAB .pdf for the use of the reader to achieve a higher degree of precision.

Charging and incubating the HAB-BART laboratory tester

The HAB-BART laboratory tester is a minimal form of the field tester including only necessary minimal steps to assure that suitable testing can be conducted effectively in the laboratory setting. When used in this environment there can be a higher level of precision due to the skills of the laboratory technologists but also opportunities to select different incubation temperatures. Procedures are similar to the field test but involve fewer steps and the differences are summarized below. Samples are dispensed into the laboratory tester using sterile pipettes under aseptic procedures. Technician error in filling the vial with water sample is reduced through the water sample being pipetted into the vial at the midpoint over the ball using a 25ml pipette. This restricts the filling error to ±0.2ml. In the event that 25ml sterile pipettes were not available then 10ml sterile pipettes cold be used with two aliquots (e.g. 10 and 5ml) being employed to charge the laboratory tester. Other procedures are addressed in DBHFWO5 For the sample coding on the individual tester it is recommended that this be done only on the top of the cap using a black permanent fine tip marker. Once dispensed, the testers should be placed in a test tube rack having a hole diameter of 26 to 28mm that would allow the testers cap to be suspended at least 90mmabove the floor of the rack giving a 10mm clearance beneath the tester. A rack should contain only a single row of testers so the activities and reactions can be more easily recognized when the rack is lifted up for observation purposes.

Protocol DBHLOO6

Using the HAB-BART (laboratory) tester to test for HAB in oil

For the purposes of this test procedure oil may be defined as any fluid hydrocarbon that has a density low enough to float on water. More reactive fractions such as those present in gasoline may compromise the polystyrene walls of the tester but fractions of diesel fuel, motor oils and some of the lighter grades of crude may be tested using this technique for the presence of HAB. This procedure uses the same procedure as DBHLWO5 but modified by the replacement of the water sample with 15mL of sterile phosphate buffer. The sequence for this test is:

1. Unscrew cap of the laboratory HAB-BART tester and place on a clean dry surface without turning over.
2. Aseptically pipette 15 mL of sterile phosphate buffer into the tester at which time the ball should float up to the fill line.

3. Dispense 0.1 mL of the oil under test aseptically over the interface between the ball and the wall of the tester. Here the oil should form a ring at the air-liquid interface.
4. Screw the cap back down firmly onto the tester and incubate with daily observations for reactions for 7 days.

There are four reactions types that need to be recorded as to the day at which they were first observed. These include the UP and DO reactions that are indicative of the presence of aerobic or anaerobic HAB. Additionally there is usually a ring of hazed plastic that forms above the air-liquid interface and gradually extends upwards if hydrocarbon degraders are present and degrading the hydrocarbons with the release of volatile daughter products that rises (when degradation is occurring by >1mm per day). This is because of reactions between the daughter volatile products and the polystyrene walls causing the haze to form. Between day 2 and day 7 there may also be a wool-like growth that forms across the base of the tester in the culturing fluids. This would mean that fungi are also active in the oil sample.

These four reactions can be interpreted as follows:

DO, HAB are present when aerobic bacteria are in the oil

UP, HAB are present when anaerobic bacteria are present in the oil

HAZE occurring above the BART ball on the plastic walls of the tester indicates a significant level of natural hydrocarbon degradation activity generating daughter products which are volatile hydrocarbons. These compounds react with the polystyrene plastic in the walls of the tester vial causing hazing. .

FUNGI, the presence of the wool-like growths generally in the base of the tester would indicate that aerobic degradation of the hydrocarbons was occurring with the fungi being involved

Protocol DBHLSO6

Using the HAB-BART (laboratory) tester to test for HAB in soil

The HAB-BART tester offers some unique characteristics that are the result of the requirement for methylene blue to be mixed into the water and the need to create a saturated oxygen (oxidative) regime. This means that an additional stage is involved in the testing of soils to allow the addition of methylene blue to the test protocol. Not only has that but soil testing required a different approach. This is because it is not possible to simply dispense the soil into the BART tester without the soil becoming perched around the ball. If this were to be done then it would also disturb the effective generation of the redox gradient essential to the functioning of the HAB-BART tester. It is therefore important to remove the ball from the tester before adding the soil sample. Addition of soil is shown in Plate three following the recommended manner in which the ball should be removed from the tester prior to the adding of the soil.

To prepare to add the soil sample for testing, the screw cap (11) has to be removed from the laboratory tester and place with the inside facing upwards. This inside surface is sterile and so should not be contaminated in any way. The ball is now rolled out of the tube into the cap (12) and the tube placed upright again. It is now ready for the soil sample to be added and the test started.

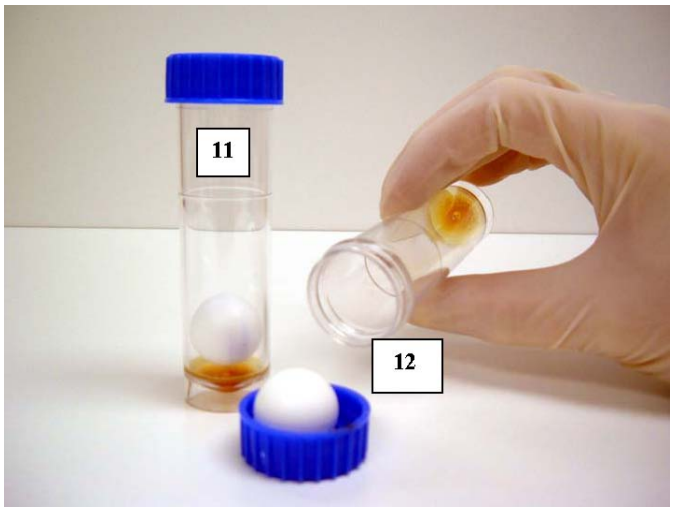


Plate Three, Removal of the ball from the tester to allow soil testing.

In plate four, the method for adding the soil to the laboratory tester is outlined to allow the start of the testing.

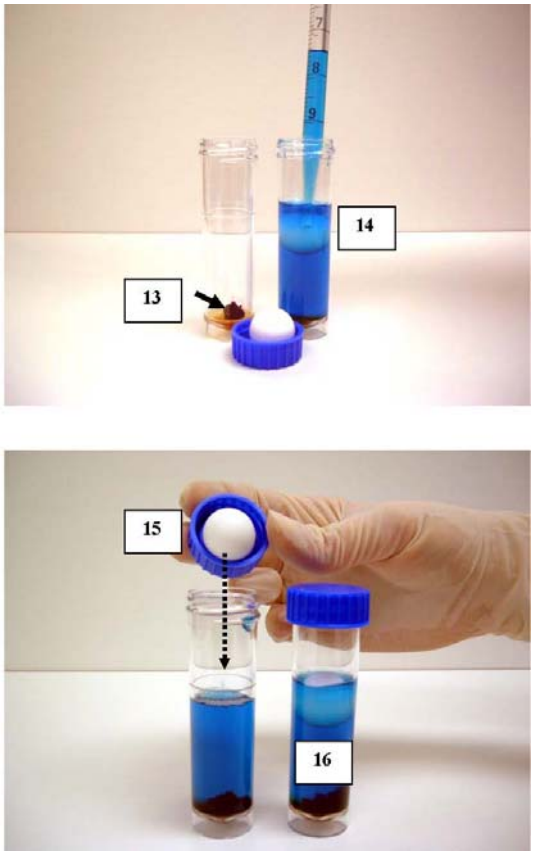


Plate Four, The addition of the soil sample to the HAB-BART laboratory tester.

0.1g of soil is added using a clean spatula to drop the soil onto the floor of the tester (13). It should be noted that a sandy soil may require 0.5g of soil to be used in order to get effective testing. If 0.1 g of soil is used then the population calculated using the HAB-BART system would need to be multiplied by x 150, if 0.5 g of soil is used then the population should be multiplied by x30 to get an accurate population count in the soil allowing for the distribution of the soil in 15ml of diluant. Once the soil has been added then 17.5 mL of sterile water is added to the second tube (16) in the soil test procedure. This tube is a second HAB-BART tester that allows the mixing of sterile water with the methylene blue to achieve an oxidative state prior to the admission of the liquid (14). To achieve an oxidative state procedure DBHSOPO5 is used on the second HAB-BART tester. Once the sterile water has been oxidized (turned blue) then the 15 mL of the oxidized methylene blue is pipetted into the tester containing the soil sample. The ball can now be rolled back into the tube (15). Once the tube is capped again (16) then the test can start at the appropriate temperature. Incubation can be at room temperature (21 to 25 ^{o}C) but other temperatures can be selected since different incubators are likely to be available. Faster reactions and activities can be achieved sometimes by incubating the testers at 27 to 29 ^{o}C but this will cause the time lags for a given population to shorten. BART QuickPop software has interpretation methods to project the population when this higher temperature is used but a summary table below gives some of the time lag to p.a.c. / ml population relationships.

It should be noted that:

1. Two HAB-BART testers are required to perform one test on a soil sample; and
2. Soil with a high fraction of oil is likely to create odd effects in the tester during incubation. When there is significant hydrocarbons (i.e., >1% by weight) the soil particles may become mobilized and float up into fluids.

Temperature Influence on Water, Soil and Oil Tests using the HAB-BART

When a soil sample is taken from a cold environment when the temperature is commonly below 8 ^{o}C for several months at a time then it is probable that the Heterotrophic bacteria may have adapted to these temperatures and will not grow even at room temperatures. In this event it is quite likely that false negatives will occur when tests are performed at room temperature. If this is a serious concern then the tests should be incubated in a refrigerated incubator at 8 to 10 ^{o}C. At these temperatures the activities and reactions are similar to those seen at room temperature but the time lag will be affected by the populations of iron related bacteria that are only able to grow at these low temperatures. For laboratory testing using the HAB-BART laboratory tester there are therefore three selectable temperature ranges for incubation (depending upon the clients needs): low temperature range of 8 to 10 ^{o}C, room temperature range of 21 to 25 ^{o}C, and the elevated temperature range of 27 to 29 ^{o}C. Temperatures above 29 ^{o}C can be used but conversion tables for projecting population numbers from time lag data are not available. This is because there is a significant influence of the constituent bacteria in the sample on the level of activity at these higher temperatures. It is therefore recommended that for higher temperatures, comparisons should be made using the time lag with conversions to population being taken as the same as for 27 to 29 ^{o}C. Conversion of time lags to p.a.c. / ml would involve different computations and these are incorporated into BART QuickPop (version 5) and are summarized in the Table three above. Note that the populations have not been corrected for soils but relate to 15 mL samples of water.

The difference in the predicted populations using these three incubation temperatures relates to the manner in which the HAB react to the temperatures. At 8 to 12 ^{o}C, the HAB that are active in those water samples are likely to be more aggressive than at room temperature and indicate higher populations. However these bacteria do not have a great deal of ability to adapt to these lower temperatures if they had been growing in warmer waters (e.g. 20 ^{o}C).Samples waters, such as those form cooling towers, hot water systems and other warm water sources cannot be easily tested using the HAB-BART tester at incubation temperatures above 55 ^{o}C due to the nature of the polystyrene materials used in the construction of the tester. The recommended upper limit for incubating the standard HAB-BART tester is 50 ^{o}C.

7. Data sheet specifications, keeping records of the HAB-BART data

It has been found that the most efficient manner to collect the data for an HAB-BART test is to uses a total of ten cells in a row to record the data generated and observed on each of the first eight days of the test. Each cell in the row would be recognized by the column as to which day in the test procedure is being evaluated:

Sample	date	1	2	3	4	5	6	7	8

Information added to the day cells is strictly limited to the two character reaction codes only. Where more than one reaction code is recognized as having occurred on that day into the test then both are entered separated by a dash. The population of Heterotrophic bacteria are calculated (using BART QuickPop) or determined using a conversion table using the day number (or hour number) from the start of the test to the first observation of a reaction. This would commonly be either UP or DO reactions.

#	Date/temp	1	2	3	4	5	6	7	8	9	10	12	14
1	mn/da/tm RT			**UP**									
2	mn/da/tm RT		**DO**										

8. Disposal methods

HAB-BART testers, when charged with a sample and incubated, are likely to contain active bacterial populations whether the tester has gone positive for heterotrophic bacteria or not. Such positive testers may be used for confirmatory tests in a certified microbiology laboratory but most would then be disposed since they are single use disposable test methods. Disposal may vary with the location of the completed testers. In the laboratory setting, the testers should be placed in a biohazard bag which would then be sealed prior to steam or gas sterilization. Once sterilized then the testers do not present a health risk issue and should be disposed of with the regular laboratory solid wastes.

In the event that the testers have been used at too great a distance from a suitable certified microbiology laboratory or do not have any arrangement to get the samples to such a laboratory then the testers do have the potential to contain active bacterial cultures. To eliminate the hygiene risks from these testers to the general society through disposal as

domestic garbage, the testers need to be disinfected or pasteurized prior to final disposal. Recommended methods for this are listed below:

Disinfection of Used Testers

Take a 10.5" x 11.25" (27 x 28.5 cms) plastic freezer bag that has a (double preferred) zip lock that can be securely opened and closed. Open the bag and place six 8.5" x 11" (22.5 x 29 cms) sheets of household paper towel which have been folded along the longer side of the sheet to make a "v" shape fold. These sheets are placed in folded side down and then opened upwards so that there are six sheets on each side of the bag. Up to 9 field testers or 15 laboratory testers can be placed in the center of the bag lying on their side (make sure the outer caps have been screwed loosened from the outer vials but the inner caps should remain tightly screwed down). Once the testers have been added then 25ml of household bleach is poured into the bag. This bleach is soaked up by the paper towel. The bag is now sealed and can be disposed of with domestic garbage. Note that the normal function in trash collection includes compressing the garbage which would cause the plastic vials to fracture and leak. There is sufficient active disinfectant in the bleach to assure the disinfection of the contents so that the risks are no greater than for the rest of the typical domestic garbage. Note also that:

- Testing has previously shown that the thiosulfate in the BART testers inactivates the bleach (unless bleach is applied in very large quantities).
- This method (bagging) is also recommended as a safe way of storing tightly capped used testers until a microwave or autoclave can be accessed.
- The bleach in the paper towel may degrade too much prior to crushing the testers in regular garbage disposal practices. Shards of plastic from the broken testers could easily puncture the plastic bag causing a potential biohazard. It is therefore recommended that the bleach be poured into the disposal bag just prior to adding the testers.

Pasteurization of Used Testers.

Heat can be employed to kill the bacteria that have grown in the tester. The recommended method involves the use of dedicated 800 to 1,000 watt microwave that would only be used for this purpose only. To perform this treatment the initial steps are the same as given above for disinfection using a microwave safe plastic freezer bag and placing the finished testers inside the bag after the household paper towel. There are two differences: (1) the testers are set up right; and (2) the caps are screwed down tightly. The microwave should be activated for 50 seconds for up to 9 field testers or 65 seconds for up to 15 laboratory testers. This amount of heat would be sufficient to pasteurize the contents and not cause sufficient distortion in the plastic vials to allow the contents to leak out and be absorbed by the paper towel. After the heat treatment then the sealed bags can be disposed with domestic garbage. Notes on the use of the pasteurization disposal technique:

- The freezer bag used must be a heavy duty one (thick Ziploc brand works well)
- Recommended microwave treatment times are -
  - 800-1200 W **50 seconds**
  - 600W **80 seconds**
- Testers should be stood upright in the plastic bag especially with the paper towel and seam on the bag as far as possible. This would reduce the risks of f leakage from the bag.
- Loosen any outer caps on testers, put in bag (hold bag upright until completely sealed), seal bag, microwave.
- Number of testers appropriate for one bag (10.5" x 11.25")
  - Labs **15 testers**
  - Field Testers **9 testers**

August 13, 2006

Appendix One

Primary Claims

The HAB-BART generates, when charged with a sample, a sufficient diversity of environments that will encourage the determination of observable activities of the HAB within the sample being tested. Two major types of HAB communities are differentiated. These are the HAB that grow in aerobic biofilms (UP reaction) and in reductive environments within anaerobic bacteria communities (DO reaction).

These claims would be subject to the following limitations:

1. The limits of detection for the HAB in a given water sample would be 67cells/L.
2. Any water sample taken for testing using the HAB-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 sample storage time to less than four hours at room temperature or twenty four hours when cooled to refrigeration temperatures.
3. The HAB-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 HAB-BART technology commonly operates at ambient room temperatures there is the ability for the testers to be used at incubation temperatures ranging from 8 to 12°C, 21 to 25°C, 27 to 29°C, 35 to 37°C and other temperatures up to a recommended maximum of 50°C under exceptional circumstances.

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