Chapter 12

Innovative BART tester Applications

Bart testers are relatively simple but durable methods for determining the activities of specific communities of microbes that create nuisance conditions within natural or engineered systems. There are almost an infinite number of applications but chapter twelve deals with some of those applications that have been used effectively. This is intended as a guide to interested readers who can see a potential use in the vast fields of microbiological challenges that can be met, at least in part, by the Bart tester systems. Using Bart testers is addressed in unusual soils, waters, and wastewaters along with other water–containing materials. This becomes challenging in the Standard Methods for BART testing since it recounts the many investigations where the results contradict the standard text book explanations. It has to be remembered that a classical text book along with concurrent regressive thinking tends to follow along narrow (linear) pathways with the reader being encouraged to never look to the side but just to follow the "yellow brick very linear road" forwards. Progress in science and technology is often made by looking sideways, forwards, upwards and downwards and listening to the real language of Nature (most important). Science described here is not recognized in text books but it does surround us everywhere that we care to look, hear, touch, and feel.

12.1 Bioremediation

Define: Environmental concerns have been raised in the last forty years particularly over the impact of recalcitrant organics such total petroleum hydrocarbons (TPH) on the potable quality of groundwaters. This has resulted in the growth using the methods commonly employed in engineered bioremediation based on the premise that oxidative processes will cause the biochemical degradation of the organics-of-concern to be accelerated. These degradative functions in bioremediation are, for the most part, bacteriological.

Apply: Of the testers it is the HAB- tester that is the most suitable for indicating these activities since it does detect oxidative (aerobic) functions as UP reactions and is effective in bioremediation processes. Here the bacterial population can become very large and active (generating short time lapses and UP reactions). If the TPH do not exceed 300ppm then the standard techniques for soil or water can be followed (see table 5.1.1.) but if there is a higher TPH then the methylene blue needs to be dissolved in the cap using 1ml of sterile distilled water and this then added after 14ml total volume in the tester has been added. This brings the total volume up to 15ml for the testing to proceed. Room temperature can be used for incubation and either the VBR I or II systems is recommended to achieve greater precision.

Consequence: Compare to control where there are no significant TPH present. Testers from the control should time lapses that are much longer than samples from the active bioremediation site and also generate UP reactions. Generating the predicted populations as pac from both the control and the active sites now allows comparisons to be made. Effectiveness may be gauged thee number of orders of magnitude increase of bacterial activity found in the bioremediation site compared to the background sampled site.

12.2 Black goop in oil

Define: Black "goop" particularly in oil pipelines originates in part from bacteria within the growing biomass located in the oil and on the surfaces are literally "mining" the water out of the oil. The color is caused mainly by the black anthracenes accumulated that growing biomass forming the "goop" (or sludges). Once the goop has formed it likes to attach to the walls of pipes particularly at over and under bends. At these sites under deposit corrosion is a very real concern. Goop (sludge) is a general term for biologically active deposits occurring within oil pipelines. It occurs as accumulates in areas of pipe over and under bends. Here the biomass inside the goop / sludge influences under-deposit corrosion (UDC) that directly impacts the long term integrity of the infested pipe wall. When UDC has become established then the typical corrosive patterns can include pitting, perforative and erosive forms of corrosion. While the former two are related to a focussed site failure (i.e. a hole in the pipe) the erosive forms of corrosion can cause the steel wall to thin and actually gain in permeability!

Apply: Aseptically take 10g of black goop / sludge and float it on one litre of sterile distilled water which contains 0.5% CB-D (a non-biocidal dispersant). Using a magnetic stirring bar agitate the oil – CB-D sterile suspension at 400±25rpm for two hours at room temperature. Water will collect under the goop / sludge and the CB-D will break up some biomass deposit. This will cause the releases of some of the bacteria from the deposit into the water primarily as biocolloidal suspendoids. Shut off the magnetic stirring and allow five minutes for the residual goop / sludge to rise to the surface of the CB-D solution. Now using a sterile 25ml pipette withdraw samples aseptically from the vertical midpoint of the available "pooled" water. Avoid recovering any black particulated material that may still remain suspended in the CB-D solution. Before proceeding on to dilution for the detection of microbiologically influenced corrosion (MIC) then it is recommended that an E-tATP (see chapter 14) be performed to ensure that there is enough bacterial activity to warrant dilution and Bart testing. Dilute 20ml of the mid-point sample with 1800ml of sterile distilled water, mix using mild agitation and then use 15ml aliquots to charge duplicate testers for the lab versions of the HAB-, APB-, SRB-, SLYM- testers. This would require 120mL of diluant. It is recommended that that VBR I unit be employed with the time lapse

set at every 15 minutes with incubation at room temperature. Using the VBR I system generates better precision for the determination of the active microbiologically influenced corrosion communities that have been recovered from the sludge / goop.

Consequence: UDC triggered by MIC is a major concern relating to over- and under bend and general associable with the biomass in the deposits, goop or sludges. Each for the four Bart testers recommended aid in the establishment of the type of corrosion that may be occurring. SRB relates to the classic sulfide producing bacteria including the sulfate reducing and the proteolytic sulfide producers. These respectively generate the BB (black base) and BT (black top) reactions. BB reactions are more likely to indicate a pitting - perforation type of corrosion while the BT is commonly more connected to the erosive forms of corrosion. Very commonly BT reactions are associable with larger biomass that may also partly function aerobically. APB communities tend to generate acidic conditions generated by the production of organic fatty acids as daughter products. Here the pH commonly falls into the 4.0 to 5.0 range and generate erosive forms of corrosion including thinning of the steel. HAB does not directly link directly to MIC but does indicate whether the bacterial community is dominated by aerobic (UP) or anaerobic (DO) reactions. The probability of an MIC event occurring is much greater when DO reactions dominate. SLYM- testing is included because this tester is the most sensitive of the Bart range and will indicate the levels of general microbiological activity occurring in the deposit, goop or sludge. Time lapses will directly relate to the size of the active community in the sample and the shorter the time lapse then the greater the predicted active population and MIC. Relative population's sizes can then be used to gauge the type of corrosion that is most likely to have been occurring at the site of interest and sampling.

12.3 Black layer in soil

Define: Soil is not even in its makeup even though at first sight it appears to lack variety. In practise soils are much subject to variability almost from one grain to the next! Soil is a very unique environment for the bacteria to grow in ranging from a commonly dry surface going though to a water-saturated base (that is in a fertile soil). Within that gradient from dry to wet to completely saturated there is also a gradient from light to dark to black as the edge of the penetrability of light is reached. For the microbes growing in such a soil gradient it ranges from dry – light through various phases of dampness – darkening to then very wet and black (devoid of light). Soils can therefore be viewed as "layered cakes" which can include an environment for every one of the microbes growing there. In a healthy soil there is commonly water draining from rainfall and snow melting that continues to recharge the soil (top down) and less commonly the water recharge comes neighbouring ground water (bottom up). Soils are therefore very important in this healthy movement. Some bacterial communities infest the "layer cake" with the effect of causing a biomass to grow laterally within the soil. This is a common occurrence but it become significant for the ongoing health of the soil when that biomass now becomes dominant in one layer causing the soil to become plugged and no allow the free flow of water. Plugging is usually caused by a lateral black layer that totally plugs the soils pores commonly between 5 and 45mm below grade. Symptoms commonly seen are water pooling at the surface and then trickling sideways to the edge of the plugging zone and then draining downwards. Coring into soils or infested golf greens sometimes shows defined lateral black layers over up to 4omm of thickness. A simple test to determine the extent of plugging layers throughout is soil is simply to throw a bucket of water (e.g. 4 gallons, 15L) where the plants do not appear to be thriving. Black plug layer is most common in sand based golf greens and be seen as patchy grass growth often more yellow than green. This is because the black plug layers are dominating over the grass roots for the water and causing the grass to die back. Combating the black plug layer involves the identification of the bacterial communities that have generated the plug and then finding methods to control that (e.g. foliar instead of soil applications of fertiliser, aeration of the soil).

Apply: Remove aseptically soil from directly inside the black layer optimally at 15 to 35mm

depths. Mix the soil into a composite sample since there will be considerable local variations. Add 15g of soil to 135mL of sterile distilled water; agitate to mix and the use 15mL charge duplicate Bart testers for the HAB-, IRB-, APB-, and SRB-. To achieve precision use the VBR I and incubate at room temperature.

Consequence: Black layer will commonly give DO in HAB- in less than two days with secondary blackening from reduced organic compounds (ROC); IRB- will give a final BL reaction in less than eight days; APB- will give a DY reaction within two days and SRB- may give a BT reaction within three days. For the four Bart tester types the probability is that the SRB- is the most significant with a BT reaction followed by the APB generating a DY. For the HAB it is common for the DO reaction to be observed but occasionally there will be an UP particularly if the sample was towards the edge of the black plug layer. IRB may generate a range of reactions with the FO, DG, and BL being the most common. If the sample happens to have been taken from the middle of the layer then it is probable that the HAB will also display a terminal BL reaction. These reactions confirm the presence of a black plug layer. Eliminating the black plug layer from, for example, a green takes time with aerification of the green being critical together with changing the fertilization pattern to a foliar feed to minimise the access that the soil microbial biomass has to those applied nutrients.

12.4 Black smokers

Define: black smokers are found in deep ocean hydrothermal vents and seeps that are exuding black hot salty water into the ocean. Water temperatures inside a smoker can reach 200 ^{o}C or more. What is most intriguing is the thermal gradients that are created around the super-heated water as it comes out of the vent and mixes with the sea water (commonly at 4^{o}C). There are some very sharp temperature changes and this very much influences the positioning of the microbial and animal biomass around the vents. Venting waters from the smoker can be high in H_2S but is also likely to contain a range of organic compounds that could then be utilised initially by the microbial biomass which then develop attached slimes and floating bio-colloids which then becomes the "feedstock" for the animal biomass which terminates with forms of shrimp, crabs and then fish. Indeed black smokers can generate a veritable menagerie in the food chain that forms around then vents. It would be very challenging to takes on-site samples given that the temperature may vary from 4 up to 350^{o}C in a nature of a couple of metres. Not surprisingly there are concentric cylinders of different organisms with each playing a specific role at various points around the vent discharges. For example, the shrimp predominantly feed on the biocolloids as it moves through the discharging waters while crabs tend to feed on slimes and smaller animals clustered on surfaces around the vent.

Apply: Bringing water samples to the surface from the deep ocean creates challenges since the sample would be subjected to reducing pressures as it is recovered. For the animals such as the crab, shrimp and fish such a depressurization would be fatal. For the bacteria such pressure drops have been found not to be fatal. For example placing pure cultures of bacteria into vials capped with 0.22micron filters and then sent down to depths of four kilometres, held for eighteen hours and then recovered showed no loss in the numbers of viable cells! These bacteria had survived not just the pressure shift but also the shifting salt concentrations and temperature changes with depth. Perhaps it is the temperature change that could be the most significant. At deep ocean depths the temperature is commonly 4^{o}C but the nature of the thermal gradients around the vents. This would mean that different life

forms could exist in close proximity to each other but would actually be functioning at different temperatures!

Once the water samples arrive on deck after being recovered from the ocean depths then there is automatically the question as to what tests and temperatures shall be performed. It needs to be remembered that the samples have travelled through changing temperature (rising), pressures (falling) and salinity (falling). Once the sample is on board then the bacteria in the sample are likely to be in a state of trauma as the cells adapt to these changes. In practise keeping the samples quiescent for four hours at room temperature may be acceptable if the tests are to be performed at room temperatures. If the testing is to be undertaken at the sampled site temperatures then this adaptation time (4 hours) should be at 4 ± 1^{o}C. It must be remembered that the normal deep ocean temperature is close to 4^{o}C. It would therefore be logical to conduct testing at both 4^{o}C (site condition) and at $22\ \pm1^{o}$C (normal room temperature on the ship). Water samples for testing do not need to be diluted (since this would create a further trauma for the bacteria in the sample). It is recommended that all testing using Bart testers use 15mL of the original sample with the preferred temperatures being 4±1 and 22 ± 2^{o}C. The VBR I system is suitable for monitoring the testers at either temperature although at 4 ± 1^{o}C the VBR I would should be installed in a refrigerated area operating at that temperature. Suitable testers for the determination of dominant bacterial communities associated with black smokers are: (1) SRB that are able to detect the presence of sulfide producing bacteria generating black sulfides; (2) HAB that would give an indication as to whether there is an adequate organic base to support growth and also if conditions are primarily reductive or oxidative; (3) APB since these would indicate fermentative activities involving the generation of acidic fatty acids; and (4) IRB that would commonly occur in the presence of iron and the ORP interfaces.

Consequence: It should not be expected that the maximum bacterial activity will occur at 22 ± 2^{o}C since the natural at-site conditions could favour 4 ± 1^{o}C. This does not preclude the possibility that optimal bacterial cultural conditions might involve a high temperature than 22 ± 2^{o}C! The unknown in this premise is the impact of the very strong thermal gradients emanating from the mixing of the deep ocean waters with the thermal waters originating from the crust. Critical potential higher incubation temperatures include 37 ± 2^{o}; 44 ± 2^{o};

54±2^{o}; and 72±2^{o}C. Within the reactions being generated the initial time lapse generated can give an indication of the activity in the community while the sequence of reactions (signature) gives an idea of the types resent and active within that sample. For the SRB there are two significant reactions but it needs to be remembered that if there is H_2S in the sample it means to be aerated for ten minutes to "remove" the gas from the sample before testing. There are two reactions. BB reaction (black in the base) is specifically caused by the sulfate reducing bacteria while BT (black top) reaction indicates reductive activity involving many different bacterial degraders. HAB detects the "organic busters" and they can be divided between aerobic (UP) and anaerobic (DO) by the reaction type. Samples close to a vent are more likely to be reductive while those taken away from the vent are likely to be oxidative. On some occasions under very reductive conditions there will be a black liquid (BL) reaction where organic carbon compounds are reduced to either elemental carbon or hydrocarbons. APB can dominate under reductive conditions causing the pH to drop into the range from (commonly 4.5 to 5.8) and stabilise. If there is sulfate or very reductive conditions then the fatty acids can be degraded with the generation of CH_4 or H_2S depending whether the ORP is below or above -150mV. IRB are commonly present and the initially generation of FO or CL reactions indicate whether the sample was taken from a primarily reductive or oxidative environment respectively.

12.5 Cancers

Define: Cancers are generally considered to be caused by tissue cells becoming unstable, and reproduce in dysfunctional manners and then dominates in the affected tissue. It is also possible that several cancers are, in reality, the product of microbial infestations within those tissues. In the last two decades there have been linkages between stomach cancers (ulcers) and *Helicobacter* species and also the human papillomona virus (HPV) and cervical cancer in women. Here there has now been developed a vaccine that provides control of these viral infections. There is an historical fallacy that developed in the twenty years from 1884 in which culture on agar based gel media was found to successfully grow many of the major bacterial pathogens. As a consequence agars became the normal operating procedures in the detection and confirmation of bacteriologically influenced infestations. However the vast majority of bacteria cannot be cultured using agar based media. Such has developed an obsession with the use of agar plates (which does generate "colony forming units") that other liquid and gel techniques were not exploited effectively. For the discovery of *Helicobacter pylori* as the cause of stomach ulcers (cancers) it was only a fortuitous event created by unfortunately heavy demands from the hospital's emergency department that left the blood agar plates incubating for five days instead of three. Those extra two days allowed the *Helicobacter* colonies to grow and be recognized. From that event then diagnostic and mixed antibiotic treatment provided confirmation and cure from the stomach ulcer infections. For the cervical cancer it was recognized that there was a virus triggering a cervical infection that then led to secondary symptoms recorded as cervical cancer. Researches revealed that a vaccine could be developed that would trigger the immune systems in a way that would prevent the initial HPV infection and then the secondary symptoms associable with cervical cancers. Today the vaccine is being widely recommended for the control of HPV infections that then also prevents those forms of cervical cancers. There are two examples of cancers thought traditionally to have originated from dysfunctional cells becoming dominant over the "normal" tissue cells and causing unfavourable effects for the host.

In the last thirty years there has developed a trend that suggests some cancers may actually be microbial infections. In the case of the stomach ulcers the "cure" is a mixed antibiotic

treatment to control *Helicobacter* species. Clearly this would indicate a bacteriologically influenced infection that can be controlled. For the control of HPV, a vaccine has now been developed which appears to control the viral infection and also the secondary symptoms traditionally recognised as being linked to cervical cancer. Other cancers may also have some significant microbiological influences that remain unrecognized due the commonplace dominance of agar cultural techniques and natural desires to get the results as quickly as possible. The next cancer that could illustrate these types of connection is colon cancer. Here there have been reports that the occurrences of active colon cancer are coincident with greater populations of sulfate reducing bacteria such as *Desulfovibrio* species. It is clearly evident that the use of an antibiotic therapy that targets a specific pathogen (e.g. *Helicobacter* species) would indicate that the dysfunction is not within the tissue cells as such but due to an infection. Similarly in the case of cervical cancer the effective application of a vaccine (specifically HPV) which prevents the development of the cancerous condition would suggest that this is also an infection now controlled by vaccination. Clearly there needs to be a re-evaluation of tissue cell induced dysfunctions leading to cancerous conditions; and the potential role of microbiological infections causing the cancer-like conditions and symptoms. This would mean re-evaluating the standard practices used in the diagnosis of cancers (as possible infections) and also the role of the chemotherapy in the treatment as being either a dysfunctional cancer cell destroyer or jus as an antibiotic controlling the microbial infection that is significantly influencing the cancer-like symptoms.

Apply: The unique cultural approach employed in the Bart testers could lead to the diagnosis of more cancers as being the product of microbiologically influenced infections in which symptoms appear to resemble cancers.

Consequence: History is already beginning to reveal the need to re-evaluate the border between tissue cells triggered cancers and microbiologically influenced infections.

12.6 Clay, from kiln

Define: Clays are dried in an oven (kiln) at high temperatures to remove most of the water and create a dry product. These kilns operate commonly at greater than 400 ^{o}C but this does not remove all of the water and theoretically kills all of the microbes. This may not be true for some of the spore forming bacteria that appear to be "protected" from these extreme heats by the clay when in the dried endospore form.

Apply: Aseptically remove cooled clay that has just gone through the kiln and place it in a sterile container. Make sure that the clay has cooled down. Aseptically take 1g samples and add to each of the following testers (use the rolled out ball into the cap technique): HAB-, SRB-, and SLYM-. Make up to 15ml in the tester with sterile distilled water. Incubate at room temperature and observe any reactions bearing in mind that the kiln-heat should have killed all of the vegetative microorganisms that were resident in the clay. In this test there may be a period during which the clay remains in suspension but this should end as the clays settles out to the base of the tester (normally less than one day). Some electrically-active clays may show particulate movements involving floating and relocation.

Consequence: If the clay kiln had been effective then there should be no detection of bacterial activity. However, if endospore formers (e.g. *Bacillus* species) did survive then there would be a delayed reaction and longer time lapse in at least one of the tester types within ten days of incubation. Note that if the surviving endospores did survive the heating process then there would be delays in the time lapse as the spores rehydrate and germinate.

12.7 Clay, separator

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

Apply: In the clay separation process there would be both liquid (usually highly turbid) and suspended solids. BART testing would be focussed on the turbid liquids using the premise that this would support the biofouling bacterial agents. The protocol would involve taking 1.5ml of the liquid sample and dispense into the tester followed by 13.5ml of sterile distilled water using the HAB- and APB-testers with the reagent in the caps (methylene blue for HAB- and bromocresol purple for APB-testers) pre-dissolved in 1ml of sterile water and then added to the charged tester. Incubation would be at room temperature with daily readings until positive or day ten.

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

12.8 Clouds

Define: Clouds form one of the major factors controlling weather whether it would be hot or cold, wet (as liquid droplets) or dry (as forms of ice). It is generally recognized that clouds do contain water droplets and generally thought that water gathered around dust nuclei (bio-nucleates). Clouds are now thought to contain water droplets formed around bio-nucleating particles which were formed from essentially active microbial cells and/or their products.

Apply: Sampling within clouds is a challenge since there needs to be a means of condensing the droplets from clouds (where they are bio-nucleated) and condensing into liquid samples. This needs to be done with a minimum disturbance to the cloud which, although large, is also very fragile while, at the same time, being dynamic. Here the most suitable method may be to float within the cloud in a dirigible (airship) and remove some the air from within the cloud using an aseptic vacuum system. Here the air inside condensers causing the droplets to condense into liquid water. Such water should be tested using 15mL samples in HAB- testers incubated at room temperature. Failing this then collecting rain during sudden storms would be equally satisfactory. Practise would dictate that it would be much easier to collect fresh rainfall in a suitable sterile container and the conduct VBR I or II testing using the HAB-, and SLYM- testers. Here the ideal would be to use the full 15mL of rain in each tester and then incubate in the VBR I system at room temperature. With the VBR II system the incubation temperature can be preselected at 28oC or if the VBR II is place in a refrigerated room at (for example 2^{o}C) then incubation can be at 6^{o} or 12^{o}C. For the HAB- tester the most likely reaction would be an UP (aerobic, oxidative) reaction if the rain is coming from white clouds but there could be DO (anaerobic, reductive) reactions where the rain sample comes from dark heavy clouds. SLYM- testers would tend to generate shorter time lapses and most commonly generate CL reactions. On some occasions the first reaction would be a thread-like slime growth forming within a part of the tester and then dispersing to a general CL reaction. Very dense clack clouds are more likely to be reductive and generate BL reactions in the lower part of the tube due to the production of elemental reduced carbon within the lower half of the tester.

Consequence: If the clouds are active with bacteriologically influenced bio-nucleation within

the droplets then an UP reaction in the HAB- tester can be expected to occur within two days. Conditions within the cloud should be highly oxidative and support respiratory forms of metabolism. In the rare event that DO reactions are recorded then this would mean the sampled rain from the cloud under stress (reductive fermentative) state and may also be less stable. In general this technique using the VBR I reveal that (heavy) rain falls contain between $20x10^6$ and $30x10^6$ pac/mL. When the RASI-MIDI has been applied using S43028 it has been found that relatively tight communities are recovered. Examination of the composition of the bacterial community reveals only a limited number of active species within the rain. This work reveals that clouds and subsequent rain falling from them are not sterile and that this supports the hypothesis that clouds are biologically dynamic. This dynamic state is possibly driven by the suspended bio-nucleated water droplets that are dominated by the intrinsic bacterial populations. In everyday language a cloud may be viewed as "Natures floating band aid" and the focal site for the removal of volatiles and suspended particles that enter the atmosphere!

12.9 Concrete, curing

Define: Concrete is viewed as a physico-chemical product that gradually hardens (cures) over time. The curing time is commonly 28days. This would indicate that possible biological functions were involved in the "curing" process as bioconcreting functions. Bioconcretions are very common in Nature where microorganisms turn predominantly inorganic structures into habitable protected sites for the incumbent biomass. Rusticles are good example of bioconcretions that are complex porous multi-layered structures in which several bacterial communities (consorms) are active but at distinctly separate locations. Concrete are different in that they ar predominantly heterogeneous mixtures of inorganics that mature into a stable modestly porous structure. Comparing Portland cement prepared under sterile conditions with a non-sterilized cement using tap water it was found that the compression strength of the sterile cement was 27MPa while for the non-sterile it was 33MPa in triplicated trials. This suggests that there are potentially significant roles for living microorganisms in the concrete curing process.

Apply: Using 1g samples of fresh concrete and SLYM-, HAB-and SRB- testers using sterile distilled water to make the volume up to 15ml with incubation at room temperature, these testers were observed daily or place in VBR I system for more precision by using the time lapse photography generating .jpg images every 15 minutes. Note that the HAB- tester system should have the methylene blue pre-dissolved in the cap using 1ml of sterile distilled water (SDW) and then added to the 1.5g sample plus 13.5ml of SDW to make a final volume of 15ml including the 1ml used to dissolve the methylene blue in the cap.

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

12.10 Drywall, sheetrock

Define: Drywall (or sheetrock) is a common construction material used for obtaining smooth finishes to internal rooms under dry conditions. Drywall is actually gypsum placed between cellulosic (paper, cardboard) layers. While the drywall stays dry then the sheets are stable. Once there are persistent damp patches then microbiological deterioration can occur. If the damp patches persist then molds (fungi) are most likely to thrive in the porous structures with spores forming on the outside of the infested drywall. If the moisture saturates the drywall to a greater extent (e.g. during a flooding of the home) then various bacteria can also biofoul the structure (e.g. initially causing weakening of the gypsum support and then collapse in the integrity of the infested part of the panel). Often it has been found that two bacterial communities (associated with the SRB- and DN- testers) have been found to actually "digest" the gypsum causing the drywall to completely collapse.

Apply: Investigations should be applied to those patches of drywall that are in states of physical collapse. This would be due to losses in structural integrity of the intrinsic gypsum within the panel of drywall. To do this, aseptically remove 1g samples of the gypsum from the drywall. Using the SRB- and DN- laboratory testers, roll the BART ball out by the standard method into the cap; add the gypsum sample; add 14ml sterile distilled water, return ball and cap. Incubate at room temperature using the VBR I system to monitor bio-generated reactions.

Consequence: SRB- tester is likely to generate a BT reaction if the drywall had become soaked in a flood including wastewater; BB reaction is likely if the drywall has become very damp for a long time but it did not involve flooding. If there is any SRB- bacterial activity then after four to six weeks the gypsum sample may be seen to disappear (bio-dissolution). However there may be black iron sulfide granules formed where the gypsum had been. DN- may or may generate foaming (FO) but will generate clouding from bacterial activities. In six to twelve weeks of incubation the gypsum sample may also disappear. Note that there is a possibility of mold (fungal) growth which will appear as black furry ring around the BART ball and there might extend over the top of the ball. Here the significant fact is that these two bacterial consorms have the ability to degrade the gypsum.

12.11 Encrustation

Define: Encrustation is the descriptor for a biomass usually growing on a surface that has a high inorganic content dominated commonly by either ferric-iron oxides or carbonates. This growth is generally heterogeneous in form, very porous and structurally resilient. These types of growths are commonly seen naturally in fractures, on surfaces over which water is passing, and in equipment such as heat exchangers. Often these types of growth can become associated indirectly with corrosion and losses in plant efficiency. This is either through the robust biomass now interfering with flows (plugging); or the interaction between the biomass deposit leading to under-deposit erosive or pitting / perforative forms of corrosion.

Apply: Sampling is relatively easy since encrustations retain form even when the water is drained out from the site of infestation after removal. It should be noted that old encrustations that have been drying out of the water environment will slowly lose their bacteriological activity. Such samples may need to be saturated with water similar to that in the natural habitat to trigger recovery. Recommended testers include the IRB- (for iron related bacteria), SRB- (for sulfide producing bacteria), and APB- (for the acid producers). Incubation is commonly at room temperature and the diluant can be either sterile distilled water (if there is a low chemistry in the water) or, in the event of total dissolved solids content, >5,000ppm then sterilised water from the original site of collection can be used. It is a common myth that high salt brines cannot support bacterial activity even in the saturated state! In reality growths can be detected for some bacterial communities such as the IRB-, HAB- and sometimes the APB- but it is rare to find SRB- active under these conditions. VBR I or II are valuable assets to monitor the time lapses and reaction types at the habitat temperature and salt concentration.

Consequence: IRB- tester is likely to generate a WB (white base of carbonates, phase one reaction) within twelve hours of the test starting if the encrustation has high inorganic carbon contents. If there is significant iron then the oxidative ferric reactions leading to one or more of the BG, BC and BR reactions are likely. If there is large mixed bacterial consorm including enteric bacteria then RC, GC are likely to occur before terminating in a

BL reaction. SRB- testers giving a BT reaction would mean that the encrusted biomass would have had high organic contents while the BB reaction would indicate that there were sulfates. If the APB- tester gives a DY reaction then that would mean that the biomass was reductive at least in part with the generation of fatty acids. Examining the surfaces under the encrustation when an iron-alloy is involved will indicate the type of corrosion. For example, shallow dishing would indicate acidulolytic corrosion by APB-. Shallow local pitting (erosive corrosion) with blackening would indicate electrolytic corrosion by SRB with BT tending to support widespread shallow pitting and BB deep pitting likely leading to perforation events.

12.12 Filter, air

Define: Filtering air is achieved using fine porous structures that entraps the solid particles (e.g. dust, mold spores) while allowing the air to pass through. Inevitably where the air is rich in particles then pores become blocked up by the particles. This causes increases in back pressure that is required to force the air through. If the filter was originally sterile or very clean then the bulk of the entrapped material would have come from the air passing through the filter.

Apply: Relatively dry air is less likely to contain bacteria but more likely to contain molds. Moist air however remains likely to contain a variety of bacteria. The most convenient way to check this is remove a part of filter (e.g. $10cm^2$) and immerse in 100ml of sterile distilled water for five minutes. Agitation should cause some of the particles to detach from the filter and go into suspension. 15ml of such a suspension could then be used in each tester (SLYM-, HAB-, and SRB-). Incubate at room temperature and observe at least daily for ten days or use the VBR I or II system. .

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

12.13 Filter, membrane

Define: Filter membranes commonly have the function of removing smaller particles such as bacterial cells. These membranes utilize pore diameters do not to allow these bacteria to pass through. Diameters commonly used are 0.45 and 0.22micron. Theoretically particles larger than those diameters should not pass through however this assumes that these particles are rigid and not flexible, Reality is that any flexibility in the cell may allow some of these particles (cells) to pass through the membrane. The pressure commonly applied to filter out the cells is -0.35Barr. Additionally smaller particles (such as biocolloids) can commonly be flexible enough to pass through under those vacuums. Particles can often collect on the downstream side of the membrane and provide nutritive environments for the survival and growth of microorganisms downstream of the membrane. It is surprising to think that there is greater biological activity downstream of the filter that has theoretically removed the bacteria! Filter membranes are considered excellent methods for trapping bacteria on the upstream surfaces of the membrane for subsequent cultural and biochemical analysis. Downstream flows from the membrane are generally considered sterile although these can have relatively high biocolloidal contents. The challenge is primarily to separate the upstream surface (trapping microorganism) from the lower downstream surfaces (gradually fouling with biocolloids and microbial infestation generated from downstream sites).

Apply: Functionally effective membrane filters should remove all viable cells of concern from the water passing though the membrane. The prime test must therefore examine this downstream water for bacterial content using the premise that the water would have been filtered and therefore theoretically should be free of microorganisms. This test can be replicated using both the SLYM- and the HAB- testers with incubation at room temperature and daily observations until positivity is recorded.

Consequence: Ideally all replicates after testing after ten days of incubation should remain negative meaning that detection limit er replicate had not exceeded 67 cells per litre seven cells per litre (67pac/L). Higher populations would be detected by one or more testers then

the types of reaction observed become critical. Of serious concern would be DO reactions using the HAB- tester and DS reactions on the SLYM- tester since these would indicate that the biocolloids are sheering from the downstream side of the filter. Here, anaerobes (DO) and dense slime formers (DS) could be present. PB and GY reactions on the SLYM- tester are also serious signals since these shows the presence of active *Pseudomonas* species.

12.14 Gypsum

Define: Gypsum is a common natural geological product composed of calcium sulfate which has a number of important uses (see 12.10 above). Under unique high salt and alkaline conditions gypsum can form as deposits that can effectively plug impacted water wells. This protocol defines the occurrences of microorganisms in the synthesis and dissolution of gypsum.

Apply: This protocol examines both the bacteria likely to be present in the gypsum and the potential for those bacteria to degrade the gypsum under reductive conditions. For this each tester would require 1g of gypsum sampled aseptically following the protocol adopted for soils. 14ml of sterile distilled water can be used as the diluant or steam sterilized groundwater from the site where the gypsum was recovered. Testers should include SRB-, DN-, SLYM- and HAB. Incubation would be at room temperature for ten days followed by weekly examination for twelve weeks for the dissolution of the solid gypsum sample placed in the tester.

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

12.15 Ice, cores

Define: Ice is considered to be frozen water that is too cold for any life to be sustained and would therefore certainly not be capable of supporting growth. Reality is that there are bacteria that can either synthesize ice even at temperatures above conventional freezing points, or produce polymeric antifreeze compounds that allow the bacteria to remain active in a liquid environment at temperatures well below freezing. Ice cores taken from "old" ice may be relatively stable for even millennia. This stability might be questionable if there has been a bacteriologically influenced intrusion into the ice that creates zones of liquid bound water within which the bacteria can remain active.. This stability in the ice cores is used to examine old ice fields to determine changes that had occurred geologically as the ice was laid down. Reality would be here that the ice would had become even "minimally" infested with microorganisms at the time of freezing then this could allow a slow but ongoing microbiological activity over time. This activity would be particularly present in zones where EPS is present along with its "antifreeze" properties.

Apply: Bacteria are capable of surviving as ultramicrobacteria, suspended animates, or spores for prolonged periods in ice let alone remaining active as vegetative cells! Ice cores are not designed to be extracted aseptically and so the outside of the core would suffer the risk of becoming contaminated. Any microbiological investigation must therefore work the central (25%) part of the core which would have been less impacted by the coring process. For the sample now maintained under aseptic conditions the temperature for thawing should not exceed 8^{o}C. This would be to minimise the temperature rise shock to any incumbent microbes that are associated with the thawing and manipulation of the sample. Once the sample is liquefied then there is a need to leave the sample for seven days to allow the bacteria time to adjust to the liquid state. All incubation of the charged testers should be at refrigeration temperatures (4 ± 2^{o}C) preferably using VBR I systems. Care needs to be taken that the testers and such fluids (e.g. diluents) as may be applied are pre-cooled to that temperature range to those temperatures to minimise cultural shock. Here even room temperatures are capable of causing a shock to the intrinsic bacteria in the ice. At least 90ml of sample needs to be collected to allow triplicate analysis using both the HAB- and

SLYM- testers. Incubate at 4±2 ^{o}C in a refrigerator needs to ensure that all of the testers have good air circulation. Examine daily for reactions for twenty eight days or until the tester generates a positive reaction.

Consequence: Because of the low incubation temperatures involved there could be extended time lapses as the bacteria adapt to conditions in the tester. There have been occasions when growth and activities have occurred in less than 24hours at these seemingly low temperatures but there could be cultural shocks which delay the initial growth. For the HAB- either UP or DO reactions are possible followed under very reductive circumstances by a partial BL (lower third to a half); while the SLYM- testers may generate CL reactions possibly preceded by TH or DS and followed by BL (lower third to a half of the tester). No correlations have yet been developed for the relationship of time lapse to the intrinsic population of potentially active bacteria in the ice.

12.16 Ice, gas hydrates

Define: Gas hydrates are deep-ocean structures embedded into the sea floor. They are dominated by an ice cap with average frozen water: natural gas ratio (H_2O: CH_4) of 1: 8±2. Complex crystalline matrices then form the gas hydrates (clathrates). These deep ocean structures form one of the largest sources of natural gas known on the planet. According to some authorities the energy reserves tied up in these clathrates is more than double that collectively found in coal, oil and natural gas wells! Clathrates appear to remain stable up to temperatures as high as 7±2 ^{o}C with the ice matrices remaining in-place. Evidence suggests that the bacterial communities involved in gas hydrates are capable of synthesizing ice at these higher temperatures. Communities that can synthesise ice at these above freezing point temperatures include some heterotrophically active bacteria (HAB-) or concretion forming (IRB-) communities. Coring into the top two meters of the clathrate allows examination of the bacteriologically influenced ice-capping on the clathrate.

Apply: Ice cores could be melted with care slowly at no higher than 12±2 ^{o}C to minimise trauma and then the incubation would be at room temperature 22±2 ^{o}C using 15ml sample volumes in HAB-, IRB- and SLYM- testers. Observations can be daily or more frequently using the VBR I system set to record .jpg images every 15minutes.

Consequence: Generally the HAB- testers generate UP reaction near the surface of the capping (0 to 100mm) and DO reactions in the deeper regions of the cored gas hydrate. However at coring depths as great as 2m there can still be oxidative zones where UP reactions then occurs. SLYM- reactions tend to be dominated by CL with follow by BL commonly in the lower third of the sample with limited FO production also occurring.

Mars has evidence of unusual surfaces structures on the surface of mars a latitude of –82.02^{o} and longitude of 284.38^{o} in a region close to the south polar ice field which could be relatable to the formation of hydrates on Mars. For example Structure A when examined appeared to be roughly circular with a diameter ranging from can be estimated 1,100 to 1,200 meters. A neighbouring structure (B) was also seen to be circular with a diameter ranging from 590 to 680 meters. Another neighbouring structure (C) had a distinctly rectangular form 490 meters long by 290 meters wide. At the time these images were taken

the sun was low on the horizon creating an emission angle of only 0.26^{o} which created long shadows over the relatively flat Martian terrain. These shadow lengths were used to determine the height of the structures since the longer the shadow then the greater the height of the object. Structure A cast an average shadow length of 65 meters indicating the height of this structure was 17cms while structures B and C, had comparable heights that were 11 and 18cms respectively. Apart from the common occurrences of gas hydrates around the continental shelf these Martian structures suggest that only the very different conditions on Mars, the possible gas hydrate structures taken on a very different (and biological) form.

12.17 Ice, glacier

Define: Glacier ice is generally ice that has formed by a packing down process (under increasing pressures at depths). This freezing would incorporate such particulate material that may have been deposited as the snow is packing down to ice as it formed. This ice is subjected to movement along with fracturing and differential melting along particulate-rich fronts. This would mean that the crystalline matrices within the ice would be more vulnerable to physical stresses than some other forms of ice. Additionally the temperature of glacier would tend to be more variable particularly if above -18 ^{o}C which could lead to a greater potential for bacterial activity. The common presence of ice worms in some glaciers could be used as indicative of bacteriological activity since the bacterial biomass which then becomes the feedstock for the worms.

Apply: Glacier ice tends to be variable in terms of physical form and color (e.g. grey, blue, black, ferric red). Greater bacteriological activity is likely to be associated particularly with these colored bands. Samples taken from glacier ice should be kept in a freezer (lower than minus 30 ^{o}C) until ready for investigation. Here the ice can now be thawed at room temperature until the ice has completely melted and then immediately tested. Incubation would be at room temperature (22±2 ^{o}C) and in a refrigerator (4±2 ^{o}C) until the tester generates complete reaction sets or for ten days. 15ml of melted ice would be used for each tester with HAB-, SLYM-, IRB- and SRB- being recommended using the VBR I system in a refrigerated room.

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

12.18 Ice, pack

Define: Pack ice essentially floats on water and is often seasonal in its occurrence depending upon the local freeze-thaw cycles and the movement patterns. This form of floating ice easily bioaccumulates material perching particularly on the underside of the ice. This becomes very significant when commonly there is a tidally influenced pack. Here ice can become exposed to pollutants and effluents originating from sanitary landfill or wastewater treatment operations. Maximum bacterial activity is generally associated with the black viscid voids that permeate the pack ice at points where there has been intrusions of these wastes. Taking an x-ray of samples from the pack ice also reveals the regions impacted by these intrusions that could also include bioaccumulated metals.

Apply: In the evaluating the bacteriology of pack ice it is important to look for signs that the ice has been compromised. Keep samples in foil or freeze resistant plastic wraps in a freezer (lower than minus 30^{o}C) until ready for investigating. To investigate particularly the blackened track ways within the ice then these can be eased out of the melting ice sample using sterile scalpels and spatulas. Incubation would be at room temperature (22 ± 2^{o}C) and also in a refrigerator (4 ± 2^{o}C) until the tester generates complete reaction commonly within ten days. 15ml of melted ice would be used for each tester with HAB-, SLYM-, IRB- and SRB- being recommended.

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

has been septic waste entrained into the pack ice.

Pack ice when it is close to human activities and particularly when close to effluent discharges from wastewater treatment plant and landfill operations. Given that the pack ice will be floating on the seawater and shifting vertically with the tides then there the potential for bio-accumulation of these effluents into the this floating pack ice. It is therefore highly probable that the ice, at least in some layers, will become impacted by the effluent biomass leading to significant changes in the nature of that ice.

12.19 Mold spores

Define: Mold spores are durable "seed-cells" that are commonly generated by fungi. These seed cells are means by which the molds can survive environments that would kill off all of the normal vegetative cells. Spores often generate very noticeable as furry growths on surfaces while the bulk of fungal biomass (the vegetative cells) remain growing unobserved in the infested relatively porous media. Mold spores are moderately resistant to heat shock compared to vegetative cells and 72 ^{o}C for 5 minutes of exposure time will eliminate much of the interferences from such vegetative activities but will allow the spores to survive. Molds are a problem under highly humid conditions such as when there is dampness with perched water patches (e.g. water-compromised drywall / sheetrock). Some fungal spores can trigger infections.

Apply: Two common sources of mold infestations are damp patches which display (commonly) black spots of growth within confined areas or in the surrounding air which does support mold spores that become suspended in the circulating air currents. For the former source then a sterile swab can be used to remove some of the growths which can then be dispersed in 15ml sterile distilled water. Spores suspended in air can be recovered by filtering the air (e.g. 1 litre) through sterile 0.45micron membrane filter. Here the membrane filter should be removed from its holder and immersed in 15ml distilled water for fifteen minutes. In either case approximately 15ml of suspension is obtained once the swab or filter is removed. Using a sterile 22 to 26mL glass tube as the holder for the suspension then the contents are heated to 72 ^{o}C for 7 minutes allowing 2 minutes for warming up the suspension. Cool down quickly by immersing the tube partially in cold water. Use 5ml of the heat-treated cooled suspension in each of two SLYM- testers with sterile distilled water and incubate at room temperature for fourteen days. Growth will occur commonly as a furry ring around the ball that may be grey or black in color.

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

12.20 Mud

Define: Mud can be considered as wet, soft earthy matter that forms on the surfaces of soils and in shallow ponded waters. Essentially muds are mixed amalgam of soil particles, clays, organics and active biomass. As such the muds can be microbiologically very active and this influences the oxidative-reductive potential (ORP), the exchange capacity of components and the associated quality of any interfacing waters and substrates.

Apply: Using Table 5.1.1 as the guide (select loam as the first option with clay as an alternative if it is known that the mud has high clay contents rendering it slippy). Testers for mud should include HAB-, SRB-, APB- and SLYM-. Incubation should be at room temperature and observations made daily or every 15 minutes using the VBR I system.

Consequence: HAB- tester will trigger as UP reactions if the mud has an open structure and DO reaction if relatively tight (and therefore likely to be reductive). SRB- will show clouding (not a recognized reaction) that could include particle cycling if there is any gas formation in the sample. High organic muds are likely to give the BT reaction and dense muds with higher sulfate content are likely to give BB reactions. If the mud is reductive and has a high organic content then APB- could give DY reactions due to fermentation with fatty acid generation. SLYM- tests will sometimes show dense slime (DS) or thread-like (TH) growths between the ball and the base of the tester. These indicate relatively oxidative conditions in the mud while a black liquid (BL) extending upwards from the base of the tester indicates very reductive conditions.

12.21 Oil, crude

Define: Crude oil consists of longer chained petroleum hydrocarbons (CxHy) which have been generated biochemically with bacteriological influences under extremely reductive conditions (e.g. 4, 01-28 OIL; 4, 06-22 BNG; and 4, 05-17 COL; Cullimore, 2010, Practical Atlas for the identification of bacteria, second edition). Crude oil along with natural gases and coals reflect the reductive stripping of the organic matter generated primarily in the surface biosphere down to elemental carbon (coal), natural gas (CH_4) and petroleum hydrocarbons (CxHy). Crude oil become a reserve that has limited stability depending upon the fractions of volatile hydrocarbons that will tend to diffuse upwards, and entrained water that then become the site for ongoing bacteriological activity. Essentially entrained water in the oil can become a "desirable" component in the oil that is then mined bacteriologically and bound within extracellular polymeric substances by the bacteria within matrices of asphaltenes. The greater the water content in the crude then the greater the potential for bacteriological activity in that oil. In old wells then the water can become so abundant that it separates out as "produced water" with inherently very high bacteriological contents.

Apply: Crude oils are predominantly oil with a small fraction of water that will sometimes increase over time. To investigate the oil for bacteriological content the following technique can be applied: (1) suspend 10mL of the crude oil on 990mL of sterile 0.5% CB-D dispersant; (2) agitate for two hours using a magnetic stir bar sufficiently vigorously to create a vortex in a 2 litre conical flask; and (3) switch off the stir bar and allow the contents to settle out for 30 minutes. If there does not appear to have been any degradation of the crude stage (2) can be repeated for a further 22 hours. Once settled then a midpoint sample should be aseptically taken to determine the enhanced total adenosine triphosphate [E-tATP commonly registered in picograms ATP (10^{-12}) per mL, pg/mL]. Methodology is described in detail in Chapter 14. If there was any significant bacterial activity within the crude oil then there would be a significant presence of E-tATP. This test now indicates primarily whether there is bacterial activity and secondarily the intensity of that activity as being suitable for Bart testing. Critical activity levels (E-tATP/mL in pg/mL) are: (i) less than 20pg/mL – no significant activity; (ii) 21 to 50pg/mL – might be a limited amount of

activity; (iii) 51 to 100pg/mL – activity detected but expect lower levels of bacterial activity using the Bart testers; (iv) 101 to 500pg/mL – moderate level of activity with one or more Bart testers detected medium levels of activity; (v) 501 to 5,000pg/mL – extensive bacterial activity detected with some Bart testers indicting very active identifiable communities; (vi) >5,000pg/mL indicates that there are very high levels of bacterial activity and it would be expected that one or more types of Bart tester will trigger short time lapses. The net effect of that is that the CB-D dispersant "opens up" the crude oil polymers allowing the bacteria to move to the sterile solution phase and be detectable by the E-tATP and then confirmed using the Bart testers. Testers for bacterial content of the dispersed crude now present in the water phase should include HAB-, SRB-, APB- and SLYM-. Incubation should be at room temperature and observations made daily or, preferably, every 15 minutes using the VBR I system.

Consequence: HAB- will commonly trigger as UP reactions in the "sterile CB-D solution" even under conditions that would suggest reductive (anaerobic) conditions exist. DO reactions can occur when there is an excess of anaerobic activity. SRB- will commonly exhibit particulate cycling within the tester solution during some part of incubation. High organic (non- CxHy) oils are likely to give the BT reaction with the oils but those with higher sulfate contents are likely to give BB reactions for the SRB- test. If the oil is reductive with high organics (non- CxHy) then the APB could give DY reactions due to fermentation with fatty acid generation but the acid reaction is likely to be buffered as the fatty acids are utilized by SRB- or CH4- (methane producing bacteria). In this case there would be a reversal to neutral pH (DYB). Generation of reduced organic compounds as black particulates are most likely to be observed in the SLYM- tester as BL (black liquid) reactions after some combination of DS, FO, CP, TH and CL (dominant) reactions. Essentially this test protocol incorporating E-tATP and then Bart testers allows an evaluation of the potential risk from the bacteria in the crude oil. These risks may be summarised as relating to corrosion and plugging (primarily as deposits that could then trigger under-deposit forms of corrosion.

12.22 Oil, machining

Define: Machining oil are generally lighter grades of oil used to work metal surfaces during processes such as lathing. Here the oil acts as a lubricant reducing friction heat and rendering more effective precise cuttings. Two problems commonly exist with the use of these oils: (1) oil will enter the atmosphere as micro-droplets; and (2) oils will combine with water to create droplets that can support bacteriological activity. The second problem is the more serious since bacteria can become very active causing:

- Degradation of the machine oil leading to losses in efficiency and precision;
- Additionally these bacteriological activities can also lead to the growth of nuisance bacteria that can cause health problems for the operator; and
- Further reduction in the efficiency of the equipment can be due to enhanced corrosion, plugging, and reduced hydraulic efficiencies.

Apply: To investigate the machining oil for bacteriological content the first step is to take a small volume of oil (10ml) and emulsify in a larger volume of sterile 0.5% CB-D in distilled water (e.g. 990ml). This oil-water mix can be emulsified using a sterile blender until the oil is dispersed in the water. Much of the bacterial biomass would now be dispersed by the emulsification process into the water phase (this is very similar to the technique used in section 12.23). After holding for 24 hours, extract 90mL taken at the midpoint of water phase under the oil emulsion for each set of testers being used. Testers for bacterial content of the dispersed oil present in the water phase should include HAB-, FLOR-, APB- and SLYM-. Incubation should be at room temperature and observations made daily or, preferably, every 15 minutes using the VBR I system.

Consequence: HAB- testers are likely to generate UP reactions relatively quickly in cases where there is significant bacteriological activity. FLOR- testers would detect the activities of significant species of *Pseudomonas* by the generation of the PB or GY fluorescent reactions later in the incubation. PB reactions are particularly significant since this would indicate a health risk to the operator. In the event the conditions within the equipment are reductive then the generation of the DY reaction by the APB- tester would indicate that a corrosion risk exists. As confirmation of the bacterial activity in the oil then the SLYM-

tester should generate CL reactions that might be preceded by DS (indicative of plugging), TH (indicating biofouling) and followed by PB or GY in the event of significant species of *Pseudomonas* being present and active in the oil.

12.23 Oil, tar sand

Define: Tar sands are very reductive localised regimes where generally heavier grades of petroleum hydrocarbons have accumulated within porous media (e.g. sand). These tar sands tend to be exothermic generating higher temperatures than the surrounding geological strata. This heat arises from the reductive (fermentative) biochemical activities (akin to composting) would be associable with the organics being reduced to hydrocarbons and possibly even reduced to particulate carbon. Tar sands have the potential to incorporate a diverse and active bacteriological biomass.

Apply: Tar sands are heterogeneous collections of sands and other media incorporating very reductive organic-rich environments that are either semi-saturated or saturated with water. In the determination of the bacteriological loading in tar sands then the first step needs to be the dispersion of the tar sand so that the active bacterial communities can be evaluated. This can be done by aseptically dispersing 10g of typical tar sand into 990ml of sterile 0.5% CB-Din distilled water. Dispersion can be done using a sterile blender with homogenisation for long enough to evenly disperse the tar sand at room temperature. Once dispersed the 1,000ml of (hundredfold dilution) suspension should be left overnight and then mid-point 15ml aliquots recovered for BART testing. It is recommended that duplicate testers be employed for the SLYM-, SRB-, IRB-, APB- and HAB- testers requiring a total of 150mL of the diluant. Incubation should be at room temperature and observations made daily or, preferably, every 15 minutes using the VBR I system.

Consequence: SLYM- testers should initially generate CL reactions that may be preceded by DS but under these reductive types of conditions there should be a terminating BL reaction that will extend from the base slowly to the midpoint of the liquid culture and then move up to under the ball. Because of the organic-rich conditions in the tar sands there should be fermentative activities leading to the DY reaction in the APB- tester which may, or may not, be buffered. HAB- testers are likely to give DO reactions rather than UP. HAB- testers are also likely to mimic the SLYM- tester and also generate terminal BL reactions. SRB- reactions are likely to be BT unless the tar sand ahs a high sulfur content which could cause the BB reaction to be generated from the sulfates. IRB- testers may generate either the

clouded (CL) or foam FO) reactions first. Here CL would indicate that the tar sand is at least in part oxidative causing respiratory forms of degradation while the FO would indicate reductive forms of (fermentative) degradation. In the latter case the tar sands may tend to generate mildly acid conditions when the daughter products (fatty acids) are present. However these would be used by the SRB- or methane producing bacterial communities depending on the ORP. If the ORP is very reductive (i.e. -150mV or more) then methane gas is likely to be generated. Where the ORP is less reductive (less than -150mV) then the SRB- communities are likely **to dominate.**

12.24 Petroleum hydrocarbons, surface leakage & groundwater contamination

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

Apply: There are three potentially impacted environments when petroleum hydrocarbons enter the ORP descending values below the grade environments. These are:

(1) Semi-saturated formations above the static water level (likely oxidative);

(2) Impacted zone at the static water level where the contaminants may have now pooled at the static groundwater interface above the saturation zone; (likely to include the ORP interface between oxidative and reductive) and

(3) Groundwater below the pooling contaminant (likely to be reductive).

Each of these impact sites could offer significant information on the bacteriological nature of the impact. For liquid samples from sites (2) and (3) then the HAB- tester would provide effective evaluation but the methylene blue would have to be pre-dissolved in the cap at the start of the test (to prevent interactions between the dissolving methylene blue and petroleum product). For porous samples from all three sites then 1.5g of sample could be dispensed following the procedure discussed in chapter 5.1. Testers suitable for this evaluation would be the SRB-, APB-, SLYM- and IRB- testers. The IRB- tester would be particularly important if there is a significant iron content (e.g. >5ppm total iron). Incubation would be at room temperature with daily readings or preferably with the VBR I with time lapse intervals set at 15 minutes.

Consequence: For liquid samples the HAB- tester should indicate whether the oxidative conditions (UP) or reductive (DO) with the time lapse indicating the level of aggressivity. In the same time run the SRB- testers would indicate oxidative activity in highly enriched organic conditions (BT) or reductive high sulfate contents (BB). APB- testers are used to indicate reductive fermentative conditions (DY) that could also cause the pH to drop into the mildly acidic range. Finally SLYM- testers would normally exhibit the activity by the time lapse to the CL reaction. Reductive organic rich environments could trigger tertiary BL reactions. Where the BL reaction occurs then it would commonly start in the base and rise rapidly up to the half way mark. In the event of an iron rich environment then the IRB- tester will become dominated by brown ferric-form reactions (such as BG, BC, BR) that are oxidative but, if reductive, then reactions would be dominated by GC, RC, FO, and BL.

12.25 Plug, black

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

Apply: Conditions are most likely to be reductive, possibly with high sulfates and/or organics. To test for significant bacteria in the black plug and sample should be taken aseptically for the investigation. It should be treated as a soil (Table 5.1.1.) and 1.5g of sample used in the testers with 13.5ml of sterile distilled water. The following testers are most likely to yield significant data: SRB- (BB, high sulfate reductive; BT, high organics potentially oxidative); SLYM-, CL indicate bacterial activity and BL would relate to reductive conditions dominating; IRB-, terminal BL reaction would indicate active iron related bacterial populations functioning under reductive conditions.

Consequence: Black plugs and slime activity would be confirmed by the SRB- tester giving either BT or BB reactions, and the SLYM- and IRB- testers generating a terminal BL reaction.

12.26 Plug, iron-rich

Define: Iron-rich plugs involve bacterial biomass dominated by ferric-forms of iron within a functionally oxidative regime present within saturated and semi-saturated porous media. Over time the biomass growth accumulates so much ferric-iron that there is hardening in the growths. Generally the iron ranges from 10% to 95% of the biomass and reflects the maturation of the plug to the point that bacterial activity becomes minimized. Once hardened the plug significantly impedes hydraulic flow creating a plugging (clogging) condition and becomes increasingly difficult to remove. .

Apply: Samples for testing should be taken as aseptically as possible and evaluation performed using 1.5g sample with 13.5ml of sterile distilled water using the methods outlined in Table 5.1.1. Recommended testers include IRB-, HAB-, and SLYM- incubated at room temperature. Here the IRB- tester is most likely to give CL, BG, BC and BR in younger samples but move to prolonged time lapses and FO, GC, RC and BL in the matured iron-rich plug. BG reactions tend to indicate that ochres may be involved in the bacterial activity. HAB- testers are likely to give UP reactions with extended time lapses in the immature plugs and shift to not detected or DO reactions in the matured plugs possibly with BL if there remains of significant organics in the plug. SLYM- testers will generate CL reactions with time lapses that can be used to assess the maturation state of the plug. For example time lapses of 1 to 2 days would mean a relatively immature plug while lapses of greater than 6 days would mean very small populations associable with a fully matured (spent) iron-rich plug.

Consequence: Maturation of the iron-rich plug is affected by localised environmental conditions (particularly around ORP interfaces) which vary with location. From the application it is possible, using the SLYM- and the IRB- tester to project the state of maturation of the plug. Longer time lapses would generally mean older and more mature (hardening) plugs with less activity particularly if there are occurrences of BL suggesting that there are local radically reductive environments within the sample.

12.27 Rain, acid

Define: Acid rain has been traditionally linked to the generation of sulfuric acid by sulfur oxidizing bacteria (e.g. *Thiobacillus*) from sulfides. In industrial regions where high-sulfur content fuels have been burnt to generate energy then the stack gases do have a high sulfide content which would become oxidized in the clouds causing declining pH in the bio-nucleated water droplets forming the clouds. In some regions where there are highly volatile organics being released into the atmosphere then there is the potential for these to become accumulated within the bio-nucleated organic-rich water droplets within the clouds. Such organics under localised reductive conditions could trigger fermentative activities with the releases of fatty acids that could also cause the pH to decline to 3.5 to 5.8 units as mildly acidic. There are therefore two likely bacteriological causes of rain: *Thiobacillus* oxidizing sulfides to sulfuric acid; and fermentative bacteria reductively generating fatty acids (e.g. APB- communities).

Apply: There is no commercially available BART tester for the sulfur oxidizing prokaryotes (SOP- tester) although one is under development in the DBI laboratories and is available for Beta testing. This is because there remains little confidence generated by any of the prototypes developed in the last fifteen years until this latest version (SOP-M series). However the APB- tester can examine rain for the presence of fermentative bacteria. At this time it is only possible to examine total bacteriological populations using the HAB- tester and the organic acidifiers using the APB- tester (reductive types of fermentative bacteria). To do this testing it is recommended that sterile vials be placed out during rain episodes to catch the rain. The sterile outer tube in the field BART testers can be used and will hold up to 70mL of precipitate. 15mL of rainwater samples would be applied to each tester (HAB- and APB-) following the standard protocol steps. Incubation would be at room temperature and reaction viewed daily or the VBR I system set up to operate every 15minutes taking visual images of the reactions.

Consequence: If acid rain is at least partly the result of fermentative bacterial activities then the pH of the rain should become acidic (e.g. pH range from 3.5 to 5.8). This would mean that if the acids were at least in part generated by reductive fermentative activities within the clouds then the APB- tester should generate a DY reaction (DY) which may be subjected to

buffering back to neutral (DYB). If this is a significant possibility then the HAB- tester would also have generate DO reactions confirming that reductive bacterial activities were dominating. Clouds with high sulfur or sulfide content are likely to be more conducive to the activities of the SOP- communities leading primarily to the generation of sulfuric acid.

12.28 Rain

Define: Rain relates to the movement of water droplets from bacteriologically influenced bio-nucleation states to free falling water droplets (rain drops). The descending rain will still contain the elements of bio-nucleation which would include the extracellular polymeric substances and viable bacterial cells. This premise considers the rain drop to be product not of nucleation around "dirt" particles (the classic assumption) but the product of trauma within the living clouds that has led to the releases of the rain.

Apply: Ideal circumstances for evaluating the bacterial flora in precipitating rain would be the entrapment of rain within a sterile container during periods of heavy rainfall. Here the deluging rain is descending in large volumes and is relatively easy to collect. Aseptic procedures need to be applied to minimise casual contamination of the pooling sample. Of the biotesters it is the bacterial (HAB-) that has been found to function with precision. Incubation can be at room temperature and VBR I monitoring is recommended (15 minute time lapse intervals). Typical HAB- populations range from 1,000,000 to 30,000,000pac/ml.

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

12.29 Scale, carbonate-rich

Define: Carbonate-rich scales have been traditionally considered to be the primary result of geochemical processes leading to the precipitation of carbonates. Recent investigations have found that bacteria functioning within bioconcretions are capable of influencing this carbonate precipitation event. While the nature of these events remains unclear they appear to occur relatively quickly. For example, the IRB- tester there can be the formation of carbonates (see chapter 3.1.2.1 for more information on the first phase WB reaction).

Apply: There are two possible scenarios for investigating the formation of carbonates using either the scale that has been aseptically removed from the sample; or as liquid samples taken when associated with some scaling event. In the event of scale the method should employ concretion from Table 5.1.1 using the IRB- iron tester. Incubation would be at room temperature preferably using the VBR I system with time lapse images taken every 15 minutes. For liquid samples then 15ml should be added to the regular IRB- tester and incubated at room temperature.

Consequence: Positive detections of carbonate synthesis within the iron biotester are the development of a white base (WB) within commonly twelve hours. Confirmation of the involvement of iron bacteria in the formation of carbonates can be achieved using sterile (autoclaved) samples. If the formation of the carbonates involves bacteria then these sterile controls should remain negative over the test period used. However if the WB reaction is related to a daughter product formation (e.g. enzyme) then the WB reaction could still occur.

12.30 Snow

Define: Snow is formed by crystallization of liquid water into solid forms as ice. This involves extracellular polymeric substances (EPS) that have been generated primarily by bacteria. This EPS now triggers the manner in which the bound water becomes frozen commonly as complex lateral plates of ice crystals. Generally these snowflakes form in a circular manner along a lateral plain in a manner that appears random. However when snowflakes are synthesised using EPS from pure cultures of bacteria then all of the crystals can often bear a common pattern. Today snow making equipment often employs cultured EPS to generate uniform types of snow. The challenge with finding the EPS "synthesiser" begins with the determination whether viable bacterial cells are still present in the snow. There is no certainty that these EPS producing bacteria will be recovered since the EPS is a product of the biomass and the viable cells no longer need to be present.

Apply: Aseptically collect one litre of snow. Keep the snow under cold conditions (freezer) until ready to attempt to detect the EPS generating bacteria. First allow the snow to melt slowly within a refrigerator ($4\pm2^{o}C$) to produce approximately 60ml of melt water. Second dispense 15mL of the sample into each SLYM- and HAB- tester (total four, two of each type and incubate one tester at $4^{o}C$, second at room temperature. Observe daily for eight days for the development of "clouding" (CL) in the SLYM- testers and UP reactions in the HAB- tester.

Consequence: Testing for the bacterial content in snow is a little "hit and miss" since while the EPS will be there, the EPS generating bacteria might not. Contrary to common sense these bacteria may grow quickly at $4^{o}C$ in the refrigerator but not necessarily at higher temperatures. If the bacteria are adaptable then $28^{o}C$ might give the faster growth. Growth would most likely be observed by clouding which is most easily observed in the VBR I system with 15 minutes set between images.

12.31 Tubercle

Define: Tubercles are ferric-rich growing "mounds" of biomass. Here the outer layers generally have the highest concentrations of iron while the central core tends to be richer in biomass. Commonly the tubercle "rests" on surfaces such as steel and there are interactions between the biomass core and the underpinning steel. This commonly leads to erosive, pitting and perforative forms of corrosion. As the tubercle matures so the bulk ferric-iron content increases and the core biomass becomes bio-concreted (hardens). In general the bulk of the bacterial activity lives within the core biomass but is likely to include iron related, sulfide producing, acid producing as well as heterotrophs. This means that the communities (3 – 15-17 TCL) are complex. In deep ocean environments the tubercles can take on the distinctive forms often seen in rusticles (3, 19-26 RST). To examine the bacteriology of tubercles there is a need to examine the biomass concretions located inside the core the growing biomass. It also needs to be recognized that as the tubercle ages then so the bacterial activity levels will decline to the point of being non-detectable within the spent tubercle.

Apply: Sampling should be limited to the core biomass in the tubercle only. This may be acquired by aseptically removing the ferric-rich outer coating (walls, layers) to expose the core which would appear crystalline, porous and commonly light yellow in color. Remove some of the core material and place in sterile Petri dish. Using Table 5.1.1 concretion analysis apportion 1.0g to each of the following biotesters: IRB-, HAB-, APB-, and SRB-. Use sterile distilled water to make up to 15ml, do not shake, and incubate at room temperature. VBR I with the time lapse setting to capture images every 15 minutes or observe daily.

UP reaction terminating as BL occurs if conditions are reductive and rich in organics. Under reductive conditions with organics there is likely to be fermentation reflected in the APB- biotester generating DY reactions that this may buffer back the dirty yellow (DY) reaction. SRB- is likely to be present if there is a sulfate rich reductive environment giving a BB reaction. In the event that conditions are more oxidative with higher organics then the BT reaction is possible if the sulfide producers are present. Commonly tubercles tend to be linked to some aspects of corrosion and the APB- and SRB- testers going positive would tend to confirm this.

12.32 Water, condensed

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

Apply: The most suitable target group for bacteriologically influenced fouling of the condenser is the HAB- tester. Aseptic samples of the condensed water should be added as 15mL aliquots into the HAB- tester and prepared following the recommended protocol. Since the water has condensed along a thermal gradient then it is probable that there will be different bacterial communities that had adapted to particular sites along the gradient. To examine the potential for HAB- to have been active at different temperatures it is suggested that duplicate testing at the incubation temperatures of 22^{o}C, 28^{o}C, 37^{o}C, and 55^{o}C. Observations should daily with the VBR I system is recommended at those temperatures with the time lapse set for the interval time lapse photography every 15 minutes. Note that the VBR II would be recommended for 55^{o}C but the VBR I could be effectively used at the lower temperatures.

Consequence: If HAB- is present it is most probable that there will be as UP reactions given that the condenser environment is very oxidative. If the condenser is heavily fouled with

biomass then there may be DO reactions indicating these bacteria were also growing under these more reductive conditions. Detection of large populations of HAB- at 37 ^{o}C, or 55 ^{o}C would indicate that the bacteria were active in the warmer parts of the condenser.

12.33 Water, deep oceanic

Define: Water forms the main constituent of the deep oceans and as such is the dominant known source of water on planet Earth. This deep ocean environment is complex built along a seawater salt gradient that can become saturated at its deepest points. Microbiologically the ocean is complex with an upper light (photic) zone which is turbulent at the surface. Here phytoplankton is the main synthesisers of the biomass. Below the photic zone, water flows tend to commonly be lateral, slow and constant. Here beyond light penetration there is a permeation of organics and oxygen from above. These chemicals are moving downwards then trigger activities in the deep scattering layer (DSL) which is one of the untold wonders of the world. Here, commonly at between 400 and 1,000 meters down into the blackness of the void photogenic microorganisms send out pulses of blue light that makes the ocean resemble a vast lit up suburbia. When descending below this zone where the "fireflies of the deep" are active then the ocean now exhibit less signs of life in the deep-ocean blackness. There are the occasional fish and squid but there are also vast clouds of bio-colloids populated by microorganisms and these persist and even become "slime" columns rising from the ocean floor where the rich sediment beds lie. Here the sediments continue to grow by gathering organics and debris as it settles. Seeping out from seafloor are natural gases and petroleum hydrocarbons along with very hot water. These gases and hydrocarbons become a prime feedstock for the deep ocean microflora. The black smokers that occur along the oceanic ridges are particularly striking (4, 03-13 BSR; black smokers) venting black sulfide rich clouds of super-heated water and feeding a rich and diverse microflora which, in turn, provides the feedstock for the animals (typically dominated by crabs and shrimp). Deep ocean environments are extremely diverse and made the more challenging by the extreme hydrostatic pressures. These pressures are, however, not particularly challenging to the bacteria active within that environment. It is possibly the salt concentration that is one of the major controlling factors. Temperature declines at depth to stabilize at around $4^{o}C$ and so this is the natural incubation temperature which actually coincides with that of refrigerators. There are therefore many different and challenging environments but this protocol will simply use HAB- and SLYM- testers with incubation at$4^{o}C$ and the main variable would be salt concentration.

Apply: There are two testers that would detect the general bacteria actively degrading organics in the deep ocean environment. These are the HAB- and SLYM- testers. Critical in the testing is the fact that all samples would employ 15ml of sample to assure that the salt concentration does not change and remains compatible to the local microflora. Incubation would be at $4^{o}C$ which would be compliant with the environment from which the sample was taken. HAB- testers would need to have the methylene blue pre-dissolved in the cap (see 3.4.1 for details). Failure to pre-dissolve causes the methylene blue to turn green (4 to 8% salt) and become completely unusable at these high salt levels. This can be corrected by pre-dissolving the methylene blue in the cap with 1mL of sterile dissolved water. Commonly it would be expected that UP reactions would occur given the oceanic environment is oxidative but if samples are taken from within bio-colloids then DO reactions may also occur. SLYM- testers are likely to give CL reactions followed by BL if the sample is from a bio-colloid and is more reductive. In the event that the sample is from an oxidative colloidal biomass then TH, DS or CP may also be generated in the SLYM- tester.

Consequence: In sequential vertical profile sampling of the deep ocean it can be expected to see radical shifts in the size and activity of bacterial populations with depth primarily relating to activity zones that are created by the phytoplankton, (e.g. DSL, biocolloids, sea floor sediments and active seeps). It is therefore less likely that there would be homogeneous populations but rather significant local variations reflecting those environments.

12.34 Water, produced from gas wells

Define: Natural gas wells are perceived to be not suitable environments for bacterial activities. This would be based on the notion that bacteria could not grow in gas (e.g. methane) under the very reductive anhydrous conditions within the well. However there is always (brackish or saline) groundwater associated with the gas well either at a distance from the well, closer towards the producing well, or coming through the perforations to flood the inside the borehole and. This groundwater can provide a suitable environment for bacterial activities which would then appear in the produced water recovered with the producing gas well. If there were significant populations of bacteria in produced water from gas wells then that could be related to:

(1) Upstream biofouling of the gas well leading to plugging and corrosion issues;

(2) Produced water acting as a conveyor system for any methane producing bacteria that might be significant contributors to the natural gas reserves; and

(3) Bacteria actually causing biofouling problems in the equipment associated with the gas well itself.

Apply: The most suitable biotester is the HAB- tester. For this test regular 15ml samples of the produced water (depressurized if collected under pressure) will work well in the HAB-tester. There is however one concern that there could be interferences from volatile petroleum hydrocarbons in the sample. When present these volatiles will react with the dissolving of the methylene blue. To prevent this then the methylene blue indicator (dried in the cap of the tester) needs to be pre-dissolved in 1ml of sterile distilled water prior to the start of the test. After one minute then the solution in the cap can be emptied into the tester to which 14ml of produced water sample has been added. Do not shake the tester but allow free diffusion of the methylene blue into the culturing fluids. Incubate at room temperature with daily observations for any reactions; or use the VBR II system at down hole temperatures. Note that salt concentrations would not be a concern since the original sample (undiluted) is used for the testing. In the event that the sample has too high a total dissolved solids content or is too clouded or colored then dilutions using the same sample are suggested. For the diluant sample then some of the sample is filtered through a 0.45 micron filter to take out the solids and bacteria and then autoclaved (steam sterilized) to

ensure sterility in the diluant. Thus the diluant would have the same salt content as the original sample for testing which would ensure that salt does not become an interference factor in the testing.

Consequence: Produced water can have a very high HAB- population often measurable in the millions pac/mL. UP reactions indicate that oxidative conditions exist. In the deeper confines of gas wells such oxidative conditions would seem unlikely but these conditions can be achieved through the use of electro-magnetic forces to protect the casing (cathodic impression) and also drive the down-hole pumps and equipment. Here such forces could then cause the electrolysis of some of the produced water generating oxygen and hydrogen. DO reactions would indicate that the conditions would now predominantly reductive. Under these conditions, there would be lower probabilities that the natural gases and volatile hydrocarbons would be degraded. DO reactions may occur when the bacterial activities may relate to fermentative activities utilizing other naturally occurring organics in both the groundwater and formation.

12.35 Water, produced from oil wells

Define: Crude extracted from oil wells are most likely to be mixtures of oil and (produced) water with the water content rising as the well appears to be becoming exhausted. Here the production of crude will decline while the produced water increases. This shift can be related to the interaction between the microorganisms in the crude and the water. Commonly water in the crude is "mined" by the microbes and bound within biomass associated with the crude. Sometimes this mining of water leads to the generation of "black goop" (see 12.3) when the water is effectively bound into the biomass. Once the water reaches higher concentrations then the water will pool within the crude as black goop. Such water produced and separated from crude is likely to have similar characteristics to the water from gas wells (see 12.34) but with a greater diversity in organic feedstock including the fuller spectrum of petroleum hydrocarbons. An additional factor that could be significant is the potential for the generated biomass to contribute to the plugging of the crude flow distribution pipe flow lines as well as at the perforations. In this event then there may be very high bacterial populations in the extracted produced water recovered from very low flows of crude from failing producing wells.

Apply: The same methodology using the HAB- biotester can be applied as described for 12.33. However there is a greater risk of secondary corrosion resulting from the bacterial activities and the SRB- and APB- testers using total volumes 15mL of water could be employed (commonly 14mL of sample or diluted sample if cloudy, and 1mL from the dissolved indicator).

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

12.36 Water, saline

Define: Water moves through a cycle from relatively pure (in rain and snow) to saline as the water moves from shallow to deep migrating down through groundwater formations. Thus there is a vertical profile with salt concentrations rising with depth extending into both the deep oceanic and crustal environments. Occasional these salt-rich waters seep up into the surface environment where the salt becomes toxic to plant and animal life. For the bacteria, there is generally a greater tolerance to salt partly because of the protective functions in the extracellular polymeric substances which keep the salt away from the cell walls. Generally salt tolerance amongst bacteria can traditionally be grouped as:

(1) Salt sensitive, cannot tolerate more than 0.01% salt;

(2) Moderate salt tolerant; generally function within the range of 0.01 and 8.0% with most bacteria becoming salt limited at 2.0 to 4.0% salt;

(3) High salt tolerance, usually function in the range of 4 to 12% salt; and

(4) Salt dependent, cannot function with less than 12% but do function in limited ranges between 12% to saturation.

In saline waters the natural salt concentration remains when 15ml of sample is applied to the tester. If dilution is required for a solid, semi-solid or porous sample then the salinity of the sterile diluant becomes critical. For the various salt ranges discussed above then the optimal salt concentration (using seawater salt would be: (1) salt sensitive, use distilled water; (2) moderate salt tolerance, 0.8% salt; (3) high salt tolerance, 8% salt; and (4) salt dependents, 14% salt. Recent laboratory studies at the DBI laboratories reveal that under high salt conditions (2 and 4 above) there is a potential for bacteria to literally create floating clouds of desalinated water. Here the "fabric" is actually the biofilm within which there is a desalination function so the water inside the "balloon" has a lowered salt content. Thus it is possible for bacteria to survive and flourish within high salt content waters by functioning solely within theses floating zones of desalination.

Apply: In the examination of the bacteriological content of these samples may use those testers that may be applicable for the perceived bacteriological problem with the fall back position being to use SLYM- testers since these have a high sensitivity to a wide range of bacteria with minimal concerns. Hitting and sampling from a desalination "balloon" is clearly a hit

or miss probability. Where there is a “hit” then it can be expected that relatively high E-tATP values (>100pg/mL) may be generated compared to the “miss” where low activities would be expected (<50pg/mL). Where there is high salinity in samples then there is a need to employ some technique to remove this salt (e.g. such as by membrane filtration, QGA technology, Luminultra, Canada) prior to the determination of the E-tATP activity. Details on the E-tATP analytical protocol is described in Chapter 14.

Consequence: This set of procedures is particularly relevant to the high salt tolerance and dependent groups (3 and 4) and may not be so significant in the low salt tolerant groups (1) and (2).

12.37 Wastewater, sanitary

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

Apply: Protocols are listed in chapters 14 and 15.