Investigation of Microbial Aspects of Groundwater Quality and Volatile By-products Related to Coal Seam Gas Development

Sampling and Analysis Plan

For

Coal Seam Gas Compliance Unit, State of Queensland
Department of Natural Resources and Mines

May 2015

Smith-Comeskey Ground Water Science LLC
Bluffton, Ohio 45817 USA

Ground Water Science
Science and Planning for Earth's Most Critical Resource

Table of Contents

SAMPLING AND ANALYSIS PLAN

This is the second work product of the Research Project entitled Investigation of Microbial Aspects of Groundwater Quality and Volatile By-products Related to Coal Seam Gas Development. This work is prepared by Smith-Comeskey Ground Water Science LLC (Ground Water Science) at the request of the Coal Seam Gas Compliance Unit, State of Queensland Department of Natural Resources and Mines (DNRM) under a Service Agreement with the DNRM.

Introduction

As discussed in the Microbial Analysis Research Project Programme Basis report (project Task 1 work product), the planned programme is aimed at:

- Characterizing the microbial ecology of sulphide generation and other expected (and perhaps unexpected) geomicrobiological processes influencing groundwater quality constituents and physical-chemical conditions in the subsurface, and formation and bore-component changes.
- Distinguishing between phenomena local to the bore (possibly due to bore ageing or poor maintenance) and "regional" phenomena (related to processes away from the bore, possibly due to CSG activities).
- In order to accomplish that goal: Developing a scientifically valid programme of testing for and interpretation of microbiological data that is both practical and valid for short-term problem diagnosis and evaluating causes underlying potential longer term trends in water quality.

This document provides a technical basis and plan for sampling bores (and other facilities as needed), conducting technically valid analyses, and interpreting results.

These procedures are not necessarily standard for the CSG application, although sampling and analytical methods are well-described in literature and some standard methods documents. Sampling will require obtaining formation water, rather than only bore-column water (both are relevant to complaints and developing product water quality). Typically, the relevant microflora occur predominantly in biofilms. As both culturing and biochemical analytical methods require the sampling procedure to process detached microflora from biofilms, it will be necessary to collect detached viable bacteria, and replicates likely will be needed.

Scope and Purpose

While it is a worthy long-term purpose to monitor microbiological changes in aquifers in contact with CSG development, it is not the CSG Compliance Unit's key focus at this stage. The Unit sees investigation of individual bore problems as the higher objective. We will prioritize the development of tools to determine whether there are microbiological causes for problems such as reduced bore yield, increasing odor and discoloration due to hydrogen sulphide (H_2S) and related products, other changing water quality, increased gas, etc., and any likely links to the CSG industry.

It is beyond the scope of this CSGCU programme to collect health-related samples, such as coliform samples. However, the CSGCU can recommend that the bore owner contact the council to arrange testing, and sample collecting times can be coordinated between agencies.

Although investigations of working bores is to be the focus, CSG Online bores are identified as good subjects for trying out enhanced microbial ecology testing methods to be used both for ongoing analysis of changes in aquifer water quality and in evaluating individual bore problems. Of a proposed 60 CSG Online bores within the program, there are at present 15 CSG Online bores completed, and 5 to 10 would be a reasonable sample of those or the full planned bore population. This selection of bores can be an excellent laboratory to refine methods practically to evaluate well problems associated with accumulating and detaching biofilms of various types, water quality change, and associated mechanisms such as microbially influenced corrosion (MIC).

Among the methods to be attempted are those intended to collect more intact or better samples of detached biofilms, and to evaluate a broader spectrum of micro-flora using cultural and biochemical analytical methods. Along with this group of CSG Online bores, we have developed a methodology for evaluation of individual domestic bore complaints. These were field-tested during our site visit exercise in April and May of this year.

Many important actors in groundwater and associated formations are not culturable. While they may not directly act on iron and sulphur compounds, they establish the environmental conditions in which these problems exist. For baseline, to understand the microbial ecology more completely, it will be helpful to collect sufficient samples for molecular biochemical analysis of the populations present, and the selected bores would be the optimal candidates.

Along with microbial sampling, basic physical-chemical water quality (pH, temperature, redox potential, conductivity, salinity, and alkalinity) would be collected.

Methods Candidates and Applications

As discussed in the Basis Report, past experience, both in Queensland and other hydrogeochemical environments that resemble the Queensland CSG fields, has identified several water quality issues and effects on bores and bore equipment that have a microbial biogeochemistry component:

(1) Occurrence of biogenic methane and CO_2 in bore water, with associated gassiness and changes in bicarbonate and carbonate saturation.
(2) Reduction in redox potential and associated stimulation of sulphate and iron reduction.
(3) Where reduced, soluble FeII or MnII are available in an oxidized water column in a bore, oxidation of the Fe or Mn.
(4) Nitrate reduction also provides a mechanism for FeII oxidation.
(5) Where sulphide is available in groundwater supplying an oxidized water column in a bore, sulphide oxidation.
(6) Biofouling associated with subjects 1 to 5.
(7) Corrosion associated with 2 and 3 and within established biofouling deposits.
(8) Tastes, odors, and discoloration associated with 1 to 7.

Thus, methods that could provide information on microbial activity on a range of redox potentials from methanogenesis to MnII → MnIV oxidation (from below -330 to > +700 MV) might be needed, at least selectively. In the four-bore sample of the field exercise, covering four distinctive hydrogeologic settings, a redox potential range of +88 MV to -199 MV was encountered. The microbes behind the generation of CH_4 and CO_2 might be assumed, unless confirmation of presence,

and information on species composition, consortia, or population numbers are needed. The necessity to include Mn oxidation or other metallic ion oxidation may depend on the geochemical occurrence or relative absence of Mn and other compounds of interest in groundwater.

Relevant Official Guidance and Standard Documents

In Australia, Standards Australia (http://www.standards.org.au/) is charged by the Commonwealth Government to meet Australia's need for contemporary, internationally aligned Standards and related services. Many standards documents are shared with New Zealand (designated AS/NZS), and may be adapted from International Standards Organization (ISO) standards.

Additionally, state agencies provide guidance and oversight. The document Monitoring and Sampling Manual 2009, Version 2, July 2013 (Queensland Department of Environment and Heritage Protection, DEHP, 2013) has been identified as a relevant guidance document in the State of Queensland for sampling and analysis of water. DEHP (2013) and relevant AS/NZS and/or ISO standards will be cited where relevant.

Standard Methods for the Examination of Water and Wastewater (currently APHA, AWWA, WEF, 2012) is widely used as a reference in water laboratories around the world, and its methods sometimes have quasi-legal status in North America and elsewhere. The *Standard Methods* standards development process is peer-reviewed, very similar to the Standards Australia process. *Standard Methods* has published sampling and analytical methods for iron- and sulphur-related bacteria (oxidizing and reducing) for decades in Section 9240. While the subject of the section is iron- and sulphur-related bacteria, methods and media for Mn-related bacteria are also included. We did not identify a comparable Australian document for these methods and so will reference Section 9240 as an authoritative source. The current edition of Section 9240 includes a selection of sampling methods, including surface collection methods, and a modern suite of physiological (culturing) methods for Fe- and Mn- oxidizing and precipitating bacteria and sulphate-reducing bacteria. It lacks reference to biochemical methods of analysis for the range of bacteria involved in Fe, S, and Mn transformation.

We likewise did not identify AS/NZS standard methods for some other microbiological parameters of interest, such as methanogens, or for biochemical analysis of microflora. Two sections of standard AS/NZS 4276 – Water Microbiology (Sections 17.1 and 17.2, sulphite-reducing anaerobes) are likely to be of interest in analysis of sulphide-affected bore water.

Biological Analyses Anticipated for Diagnostic and Monitoring Testing

Microbiological methods for biofouling and biocorrosion testing

The following methods can (at least partially) answer the following questions:

- Is biofouling present and biocorrosion active?
- What types of biofouling and biocorroding organisms and activities are present?
- Is the bore or associated systems more or less affected than before? The answer to this last question requires monitoring over time.

For bore maintenance and problem diagnosis purposes, methods that provide rapid, general insight into biofouling and biocorrosive conditions (what is happening?) are generally preferred over methods that characterize genetic make-up or metabolic capabilities (who is doing it and how?).

(1) Examination by light microscopy: This has traditionally been the method of choice for confirming and identifying components of biofilms (APHA, AWWA, and WEF, 2012, Section 9240). However, in many instances, biofouling as a cause of well problems may be difficult to diagnose via microscopy alone, even with very good tools and skills (Smith, 1996) and identification by microscopy alone is prone to error.

(2) Morphological examination of components: Components from bores and water systems can also be examined to give a presumptive diagnosis of MIC and biofouling.

(3) Cultural enrichment and physiological indication: Culturing can provide a means of detecting non-filamentous, metabolically active biofouling and MIC microflora, and also to profile the ecological physiology niches occupied by microorganisms. Among the available methods in the cultural approach for routine maintenance monitoring purposes is the BART™ Method developed by Droycon Bioconcepts Inc., Regina, Saskatchewan, Canada (Cullimore, 2008; Smith, 1992; Smith, 1996; Smith and Comeskey, 2009). This method was found by Smith (1992) in field trials to provide useful qualitative information in well biofouling events and is increasingly accepted as a standard biofouling monitoring method. A similar system, employing bottled liquid cultures, is marketed by Laboratorio MAG in Argentina (Gariboglio and Smith, 1993; Smith and Comeskey, 2009). Both BART and MAG iron-related and sulphate-reducing methods are now included in the 22nd Edition of Standard Methods Section 9240. Using Section 9240, a facility's laboratory can also prepare its own culture media for the same purposes, as well as for for Mn- or S-oxidizing biofouling.

The advantage of the commercial test products over laboratory-formulated tubes and plates include:

- Consistent formulation and shelf life (especially in the case of the dry BART tubes)
- Lack of need for the CSG Compliance Unit to invest in microbiological laboratory preparation capacity and facilities
- Lack of need for incubation and similar laboratory facilities
- Portability for field use, including inoculation in the field
- Liquid incubation is more realistic than the plate environment for aquatic bacteria
- Commercial support for use and interpretation.

Because of supply practicality, we recommend use of the BART method over MAG and over laboratory-formulated media due to the lack of permanent environmental microbiology facilities and professionals with the CSG Compliance Unit.

The BART method tubes come with a variety of media mixtures. The IRB-BART, for example (Section 9240) is designed to recover anaerobic (sulfur- and nitrate-reducing) and microaerophilic heterotrophic Fe-precipitating microorganisms. The SRB-BART (Section 9240) effectively recovers sulphate-reducing bacteria. Smith (1992 and 1996), Smith and Comeskey (2009) and Cullimore (2008) provide guidance in BART method use. Both the BART and MAG media are based on published media formulations.

The IRB-BART has poor recovery for Mn biofouling, and really need to be supplemented by a Mn-related selective enrichment medium if such analysis is deemed necessary. Likewise, there is no S-oxidizing BART or MAG enrichment medium. Some users, including the author of this report (Smith, 1992; Smith-Comeskey Ground Water Science, 2012) prefer to combine methods for iron, sulfur, and manganese biofouling analysis.

There are also BART media in addition to the IRB and SRB models. These are not included in *Standard Methods* but have been reviewed by Canada Standards for quality assurance. For example, the DN-BART selectively enriches for denitrifying bacteria. Denitrification is an important pathway for FeII oxidation and the process is common in aquifers. As described in Smith and Comeskey (2009) and Smith-Comeskey Ground Water Science (2012), selecting BART methods covering a range of redox potential conditions provides a field-usable means of assaying the microbial ecology of a system of interest, such as a water bore.

For evaluation of MIC potential, SRB-BART will be used to identify sulphate-reducing (typically corrosive) environments. If certain drilling fluid products have been used in the bore in question, the AS/NZS 4276.17 tests for sulphite reduction can be used to supplement. The Hach PathoScreen method appears to meet this standard and has been selected by the project for this purpose. Certain denitrifying conditions identified by DN-BART are corroding. IRB-BART reactions (see following) can be a secondary indicator of reductive MIC. The APB-BART (selecting for acid-producing bacteria) can round out likely causes of MIC of bore equipment.

Interpretation of BART and PathoScreen: These tube-based tests do not permit the direct visual count that can be made using heterotrophic plate count methods. Instead, BART are evaluated for both 1) the type of reaction and 2) the time until the reaction occurs. The type of reaction can be interpreted to provide some idea of the bacteria present. The time until the reaction has been empirically compared to the colony forming units (CFU) per mL count developed to evaluate plate count results. So a certain “day to development” of a reaction is analogous to a CFU/mL count. These can be compared over time in long-term monitoring (Cullimore, 2008; Smith and Comeskey, 2009). PathoScreen tubes are incubated for 24-48 hr, and a positive reaction is a change from a clear, yellow colour to black.

Culturing for methanogens and methanotrophs

As discussed in the Task 1 Basis report, there are culturing methods for methanogens. However, it is clear in reviewing the procedures that conducting such culturing is much more challenging than culturing the above-mentioned types of interest. For the present, we anticipate using the presence of methane and carbon dioxide (or shifts in carbonate geochemistry and alkalinity that can be attributed to enhanced CO_2) as surrogates for the presence of methanogens and methanotrophs, with baseline confirmation by biochemical means (as discussed in the following).

Biogeochemical Methods

These methods were discussed conceptually in the Task 1 Basis report. We are recommending the use of the quantitative PCR (qPCR)-based method, as employed by Microbial Insights Inc. (Knoxville, Tennessee, USA, and Urrbrae (Adelaide), S.A., www.microbe.com) in the QuantArray form as employed by Microbial Insights, or a substantially comparable service in Australia. Such methods

have a track record of use in groundwater studies, including saline water (e.g., Missimer et al., 2014; Smith, 2015).

The QuantArray-MIC analysis subdivides samples and analyzes for specific genetic markers, and analyzes for the following table (next page). An alternative would be to choose methods "a la carte" in the CENSUS format as discussed in the Task 1 Basis report. As Microbial Insights explains it, the number of analyses in this method is fixed and cannot be reduced. A policy decision would be to consider if we only need a smaller number of identifications, for example, for methanogens and methanotrophs, and employ CENSUS for that. The table:

QuantArray-MIC qPCR Functional Analytical Targets (http://www.microbe.com/quantarray-mic/)

Target	Relevance / Data Interpretation
Total Eubacteria	MIC is initiated by growth of a biofilm on the material surface. Monitoring total bacteria provides a general measure for evaluating bacterial growth.
Total Archaea	Depending upon types and environmental conditions, total archaea can outnumber total bacteria and be a more important factor in MIC.
Sulphate Reducing Bacteria	SRB consume hydrogen, produce hydrogen sulphide and are the microflora most commonly implicated in the pitting corrosion of various metals.
Sulphate Reducing Archaea	Sulfate reducing archaea (SRA) consume hydrogen, produce hydrogen sulphide and have been implicated in MIC at elevated temperatures.
Exopolysaccaride Production	Targets genes involved in the production of exopolysaccharide (EPS) and biofilm formation by some *Burkholderia* spp.
Methanogens	Methanogens utilize hydrogen and can contribute to cathodic depolarization and can cause corrosion rates comparable to SRB.
Fermenting Bacteria	Anaerobic bacteria produce organic acids and hydrogen. Acid production can lead to localized drops in pH facilitating corrosion while supporting methanogens and SRB.
Nitrate Reducing Bacteria	The qDNF assay quantifies target genes encoding enzymes responsible for a key step in biological nitrate reduction (which can result in FeII → FeIII oxidation).
Acid Producing Bacteria	Acetogenic bacteria are strict anaerobes that produce acetate from the conversion of H_2-CO_2, CO, or formate, supporting methanogens. The presence of acetic acid is known to exacerbate CO_2 corrosion of carbon steel.
Iron Oxidizing Bacteria	Iron oxidizing bacteria are a group of microorganisms commonly implicated in metal deposition and tubercle formation. FeIII oxides aggregate As.
Manganese Oxidizing Bacteria	Like iron oxidizing bacteria, manganese oxidizing bacteria are capable of making deposits of metal oxides.
Sulphur Oxidizing Bacteria	Often aerobic bacteria oxidize sulphide or elemental sulphur producing sulphuric acid and biofouling.
Iron Reducing Bacteria (three assays)	Iron reducing bacteria reduce insoluble ferric iron to soluble ferrous iron potentially facilitating the removal of protective corrosion products formed on exposed iron alloy surfaces and increasing total Fe in water. Three assays targeting 1) Deferribacter, Ferrimonas, Geopsychrobacter, Geothermobacter, Geothrix, Geovibrio, Geothermobacterium and Albidiferax, 2) Geobacter, 3) Shewanella.
Iron Reducing Archaea	Targets two genera of iron reducing archaea, Ferroglobus and Geoglobus.
Nitrogen Fixing Bacteria	Nitrogen fixation converts nitrogen gas into ammonia.
Ammonia Oxidizing Bacteria	Ammonia oxidation or nitrification produces nitric acid causing. Depending on alkalinity levels, nitrification in water systems can increase lead contamination and increase copper solubility.
Deinococcus spp.	Genus of bacteria considered very efficient primary biofilm formers and therefore have been implicated in slime formation and biofouling.
Meiothermus spp.	Like *Deinococcus* spp., *Meiothermus* spp. are efficient primary biofilm formers and frequently implicated in slime formation and biofouling.

Biochemical methods have the advantage at some balance point of being *cost-effective*. Investment in culturing infrastructure for some microflora such as methanogens involves significant cost. Sampling for these is specialized as well. Packaged cultural methods recommended above also have costs for purchase and sanitary disposal after use. The alternative to packaged tests such as BART is custom formulation, requiring laboratory infrastructure, although test formulation can be contracted. Sampling and sample processing for biochemical methods is also relatively simple and does not require infrastructure development.

Coordination with physical-chemical analyses

As discussed in the Task 1 Basis report, microbiological testing should be coordinated with and interpreted in light of physical-chemical sampling and analysis to diagnose bore problems and in monitoring microbial changes in groundwater (Smith 1992, Smith-Comeskey Ground Water Science, 2012; Smith and Comeskey, 2009), including water levels or artesian pressure in the bore. Water quality histories permit the identification of change in parameters that can be attributed to mechanisms such as biofouling. Analyzing physical-chemical water quality data can also provide insight into aquifer and bore redox potential, and by extension, biogeochemical activities within the capture zone of the bore of interest (Jurgens et al., 2009). In the case of the present study, sampling and analyzing gas and volatiles in bore water is of particular interest.

The CSGCU has developed a flow cell device for optimizing on-site physical-electrical water quality data collection, analyzing for conductivity, pH, temperature, and redox potential using electrode methods and the flow cell.

Biofouling and organic solids: Analyzing the nonmicrobial components of biofilm samples (e.g., deposits on pumps or pipes) is highly useful for identifying the biofouling and biocorrosion processes at work in and around a bore of interest. Where biofouling samples containing solids are collected and analyzed, it is useful to examine these by light microscopy, for Loss on Ignition (LOI), a surrogate for organic matrix (extracellular polymer or slime), and inorganic materials of interest, typically carbonate, sulphate, and metal oxides and sulphides.

Summary of relevant physico-chemical parameters in bore microbial ecology analysis

Fe (total, Fe^{2+}/Fe^{3+}, Fe minerals and complexes), Mn (total, Mn^{4+}/Mn^{2+}), minerals and complexes:	Indications of clogging potential, presence of biofouling, Eh shifts. Fe and Mn transformations are the most common among redox-sensitive metals in the environment. A shift in redox couples toward oxidation usually accompanies oxidative biofouling. A persistent notable (no set value) rise in values usually goes with redox reduction and/or increase in organic carbon. Transient spikes in Fe usually indicate corrosion, often biocorrosion.
S (total, $S^{2-}/S^{0}/SO_4^{2-}$, S minerals and complexes):	Indications of corrosion and clogging potential, presence of biofouling, Eh shifts. Appearance of sulfide, often with a reduction in total Fe indicates presence of SRBs. Some $S^{=}$ production is sulfite reduction degrading drilling fluid products. Clogging or slime in an absence of Fe, often light-coloured, indicates sulphide oxidation, typically biological. All sulphide production in potable aquifers and oil and gas reservoirs is biological.

Ammonia and nitrate-nitrite	Indications of organic clog build up, changing WQ conditions. All shifts in N species total values or ratios are microbially mediated.
Eh (ORP or redox potential):	Direct indication of probable metallic ion states, microbial activity. Usually bulk Eh, which is a composite of microenvironments. A notable drop in Eh or ORP may indicate biofouling, but not necessarily. Oxidation shifts are less likely to be microbially mediated.
pH:	Indication of acidity/basicity and likelihood of corrosion and/or mineral encrustation. Combined with Eh to determine likely metallic mineral states present. Rarely affected by microbial action.
Conductivity:	Indication of TDS content and a component of corrosivity assessment. May rise sharply due to biocorrosion. Certain components of TDS such as chlorides, sulphates, magnesium, calcium and carbonates affect corrosion or encrustation in water conveyance systems. Combine with Fe changes, and compare bore column and formation values.
Major ions:	Carbonate minerals, F, Ca, Mg, Na, Cl determine the types of encrusting minerals that may be present and are used in saturation indices. One surrogate for many cations is total hardness and alkalinity for anions. Microbial oxidation of CH_4 to CO_2 shifts by microbial action cause rise in alkalinity.
Turbidity:	Indication of suspended particles content, suitable for assessment of relative changes indicating changes in particle pumping or biofouling. Use of the “small balls” test (allowing a turbid solution to settle and observe for spherical colloids that indicate biofouling) is a practical and reliable field test.
Sand/silt content (v/v, w/v):	Indication of success of development/redevelopment, potential for abrasion and clogging.

Should ion-specific field analyses for metals, such as those developed by CSIRO for Fe^{2+}, Fe^{3+} and Cu (e.g., Vepsaelaeinen et al., 2014) be available and seem to the CSGCU to be relevant to an investigation, these can be added.

Sampling for Analysis

In this current investigation, sampling from bores is the major focus. Thus sampling would largely be focused on obtaining water samples, biofilm samples from water-affected systems, and the occasional more massive solids sample. Most of the analyses discussed are focused on water, but can be used for solids analysis. We anticipate using the following methods.

(1) Time-series sampling: In this approach, the pump is allowed to shut down for a period of time from 2 hours to several days, and then replicates of samples are collected at each sample event. This procedure helps to overcome the statistical limitations of pumped grab sampling for cultural analysis. Samples for biochemical analysis (if collected) would be collected in the middle of the procedure. Water levels or artesian bore pressure and flow rates are recorded as feasible.

(2) Surface collection: Grab samples remain unreliable for microscopic and biofilm-solids analysis (Smith, 1992; Tuhela et al., 1993). We anticipate using a variation on the flow cell system in Smith

(1992) as described in Section 9240 in the 22nd Edition of Standard Methods. Coupon sampling apparatus developed for MIC evaluation may also be used.

(3) Necessary adaptation: As discussed in the Task 1 Basis report, as needed, sampling protocols will be modified to accommodate the limitations of sampling points, with variations from written protocol detailed.

A detailed sampling protocol description follows in Appendix A. Sampling will generally follow the recommendations of DEHP (2013) and AS/NZS 5667 and supporting standards. Special focus will be on aseptic technique intended to minimize or eliminate contamination by microorganisms not part of the intended sample. All samples must be uniquely labeled and identified per a DNRM-approved protocol, geolocated, and samples and results recorded together. Typically, sampling events will be photo- and video-recorded digitally.

Minimum Data Elements

At a minimum, an ongoing monitoring program for wells should include a one-time use of a relevant biochemical suite, and the use of tests kits (BART and PathoScreen) and other self-monitoring (biofilm collection and visual inspection of components). BART testing may be used in a one-time format, but should be relatively frequent (quarterly) for ongoing monitoring (as distinguished from one-time diagnosis). Biofilm collecting can be conducted in a baseline troubleshooting role and then annually or at observed changes.

A quality assurance and quality control (QA/QC) programme meeting the standards of the DNRM, AS/NZS Standards 2031, 4276, and 5667, and the microbial ecology community should be in place. The QA/QC procedures and standards of APHA, AWWA, WEF (2012), including Sections 9020 to 9060, and DEHP (2013), necessarily adapted for the present purpose, can provide guidance (among other references).

Summary of the Sampling and Analysis Program

1. Select 5 to 10 bores from CSG Online inventory of monitoring bores for method development, depending on budget and practicality.
2. Conduct time-series pumping testing on 5 to 10 bores to collect microbiological samples for the following analyses. As often as agreed to under the programme.
3. Conduct parallel on site physical-chemical analyses and collect desired samples for physical-chemical analysis as part of the larger CSG monitoring programme. Record static (nonpumping) water levels and artesian pressure, pumping water levels at sampling events, and flow rates during sampling. Observe and record (on sample record and on visual media) observations on odor and physical parameters (e.g., gassiness) and appearance of water and the bore.
4. Conduct baseline biochemical (one time) analysis on 5 representative of the 5 to 10 using ideally a) QuantArray inventory or if budget does not permit b) 5 CENSUS markers selected to fill weak spots in available cultural methods. Repeat as desired. Process and send to analytical laboratory as instructed by the laboratory.
5. Conduct parallel culturing for IRB-, SRB-, DN-, APB-, HAB-BART, PathoScreen, and if groundwater quality warrants, add a Mn-oxidizing enrichment test (Section 9240). Collect

samples at each stage in time-series sampling using aseptic technique (Appendix A). Samples will typically be split into the 15-mL subsamples used to inoculate a BART tube. Inoculated samples should be carefully transported to the facility where they will be observed. Observe at least daily at the same time and under consistent conditions and record changes. Document appearance by description and photograph.

6. In a sulphide-affected well where drilling information suggests it is relevant, collect samples for sulphite-reducing bacterial analysis (AS/NZS 4267.17), PathoScreen. Collect at second stage of time-series. Analyze for presence/absence and population (by plate count or MPN) if warranted.
7. On selected bores (3 to 5) install biofilm collectors and trickle flow through them for 1 to 2 weeks. Recover slides and other coupon surfaces for observation and analysis of solids.
8. Methods demonstrated and used to document the microbial ecology and related geochemistry of CSG Online bores will be selected for diagnostic testing of water supply bores potentially affected by CSG activities. Not all methods would be used, but those that are judged necessary based on the circumstances.
9. Among the QA/QC methods will be inoculating selected BART (usually HAB-BART unless there is a purpose in using another) with sterile water from containers and other equipment touching bore sample water. Randomly selected, freshly opened BART tubes (and other tubes as used) will be inoculated with sterile water and used as transport blanks. Sample containers for collecting microbiology must be sterile and to the extent possible meet the requirements of AS/NZS 2031. BART are typically (unless ordered as “Lab BART”) supplied with a gas-sterilized sampling bottle, which will be used wherever possible.
10. All sample identification and associated results will be recorded in raw form and made available per CSG Compliance Unit procedures, and analyzed and interpreted professionally.

References Cited/Bibiography

Australian/New Zealand and related ISO standards

AS/NZS 2031. Selection of containers and preservation of water samples for microbiological analysis

AS/NZS 4276.1. Water microbiology - General information and procedures

AS/NZS 4276.17.1 and AS/NZS 4276.17.2. Water microbiology - Method 17.1: Spores of sulfite - reducing anaerobes(clostridia) including Clostridium perfringens - Membrane filtration method and Water microbiology - Method 17.2: Spores of sulfite - reducing anaerobes (clostridia) including Clostridium perfringens - Estimation of most probable number (MPN) using the multiple tube dilution technique.

AS/NZS 5667.1. Water quality - Sampling - Guidance on the design of sampling programs, sampling techniques and the preservation and handling of samples

AS/NZS 5667.11. Water quality - Sampling - Guidance on sampling of groundwaters. Technically equivalent to and has been reproduced from ISO 5667-11, Water quality -- Sampling -- Part 11: Guidance on sampling of groundwaters.

ISO 5667-14. Water quality -- Sampling -- Part 14: Guidance on quality assurance and quality control of environmental water sampling and handling.

ISO 5667-22. Water quality -- Sampling -- Part 22: Guidance on the design and installation of groundwater monitoring points.

ISO 19458. Water quality -- Sampling for microbiological analysis.

Literature

APHA, AWWA, WEF, 2012. Sections 9020 to 9060, addressing QA/QC, *Standard Methods for the Examination of Water and Wastewater*, 22nd ed. American Public Health Association, Washington, DC. Online 2012, subscription required (some online material may be newer), www.standardmethods.org/.

APHA, AWWA, WEF, 2012. Section 9240 - Iron and Sulfur Bacteria. *Standard Methods for the Examination of Water and Wastewater*, 22nd ed. American Public Health Association, Washington, DC. Online 2012, subscription required (some online material may be newer), www.standardmethods.org/.

DEHP, 2013. The Monitoring and Sampling Manual 2009 Version 2 July 2013, State of Queensland, accessed at https://www.ehp.qld.gov.au/water/pdf/monitoring-man-2009-v2.pdf February 2015.

Cullimore, D.R. 2008. *Practical Manual of Groundwater Microbiology*, 2nd ed., CRC Press, Taylor & Francis Group, Boca Raton, FL, 376 pp.

Jurgens, B.C., P.B. McMahon, F.H. Chapelle, and S.M. Eberts, 2009. An Excel® Workbook for Identifying Redox Processes in Ground Water, Open-File Report 2009–1004, U.S. Geological Survey, Sacramento, CA, accessed January 2015 at http://pubs.usgs.gov/of/2009/1004/

Missimer, T.M., C. Hoppe-Jones, K.Z. Jadoon, D. Li, S.K. Al-Mashharawi, 2014. Hydrogeology, water quality, and microbial assessment of a coastal alluvial aquifer in western Saudi Arabia: potential use of coastal wadi aquifers for desalination water supplies. Hydrogeology Journal 22, Issue 8, pp 1921-1934.

Smith, S.A. 1992. Methods for Monitoring Iron and Manganese Biofouling in Water Supply Wells. AWWA Research Foundation (now Water Research Foundation), Denver, CO.

Smith, S.A. 1996. Monitoring biofouling in source and treated waters: status of available methods and recommendation for standard guide. *Sampling Environmental Media*, ASTM STP 1282, American Society for Testing and Materials, West Conshohocken, PA, pp. 158-175.

Smith, S.A. 2015. Hard Hat Geomicrobiology: Using biochemical and physiological microbiology methods to solve practical groundwater supply issues (abstract submitted to the 42nd Annual IAH Congress), International Assn. of Hydrogeologists.

Smith, S.A. and A.E. Comeskey, 2009. *Sustainable Wells: Maintenance, Problem Prevention, and Rehabilitation*, Taylor & Francis CRC Press, Boca Raton, FL.

Smith-Comeskey Ground Water Science, 2012. Wellfield Operations and Maintenance Water Quality Testing Manual, available at www.groundwaterscience.com, Smith-Comeskey Ground Water Science, Bluffton, OH USA.

Smith-Comeskey Ground Water Science, 2015. DRAFT Microbial Analysis Research Project Programme Basis, prepared for Coal Seam Gas Compliance Unit, State of Queensland Department of Natural Resources and Mines, Toowoomba, Queensland, March 2015.

Tuhela, L., S.A. Smith, and O.H. Tuovinen. 1993. Microbiological analysis of iron-related biofouling in water wells and a flow-cell apparatus for field and laboratory investigations. Ground Water 31(6):982-988.

Vepsaelaeinen, M; Chen, M; Yang, Y., (2014), Cu^{2+}, Fe^{2+} and Fe^{3+} analysis of bioleaching solutions using chronoamperometry and BDD electrode. Journal of Applied Electrochemistry, 44, 1135-1143.

Appendix A: Step-by-Step Sampling Protocol

Personnel: Two CSGCU field staff, one focused on instruments and the other on microbiology, but working together

Typical equipment:

Dedicated disinfected hose and fittings

Flow cell apparatus developed by CSGCU and a range of attachments to match bore discharge arrangements

Field meters for conductivity, temperature, pH, and redox potential (oxidation-reduction potential, ORP), and gas detector equipment

Sample bottles for water quality, sterile sample collection containers for microbiology, gloves

Microbiology tubes to be inoculated in the field (e.g., BART, PathoScreen), transport blanks for microbiology (BART and PathoScreen inoculated with sterilized water)

Containers for biological tube transport

Typical pumped water sampling plan:

1. If possible, have the bore be switched off for several hours.

2. Detach existing hoses if any from the sample tap and clean and disinfect the tap.

3. Attach dedicated hose and connect water quality flow cell and any flow bypass to manage flow rate.

4. If possible, measure water levels and flow rates.

5. Install electronic water quality meters and start flow.

6. Once flow is adjusted to avoid flow cell overflow, take a microbiological sample from the sampling tap on the flow cell. Use aseptic technique. Mark sample as Sample 1 or FF (first flush) for the site and set aside for processing. Note date and time. If water is visibly turbid or biofouled, take additional samples.

7. Take and record water quality readings until conductivity and ORP stabilize (ORP may cycle back and forth across a central reading). If stabilizing before a bore volume is pumped, pump until the calculated bore volume is pumped off

8. Photograph the scene for the record.

9. Upon stabilization, again using aseptic technique, collect a second set of microbiological samples, labeling as before, Sample 2 or LF last flush, with date and time. This step may be repeated as desired.

10. Inoculate BART and PathoScreen tubes at the bore site if possible, following manufacturer instructions. If possible, split sample among tubes. Alternative: with very low flow rate (and in the absence of blowing dust), BART tubes may be inoculated directly from the flow with care. As PathoScreen is especially sensitive to hand contamination, wipe media packets with alcohol and use alcohol or flame-disinfected scissors to cut the packets, instead of fingers.

11. No need to cool samples or incubated tubes unless air temperature is well above water temperature. Keep BART samples upright, as still as possible, and out of sunlight.

12. If taking water samples for biochemical (such as DNA) analysis, follow receiving laboratory instructions exactly.

Alternative sampling protocols:

1. Pumping alternatives: A set of multiple samples can be collected in time series as discussed elsewhere in this document. Samples may be collected during steps of a step-drawdown test. If using an alternative sampling protocol, describe the methodology for your report.

2. Installing a biofilm collection flow cell (distinct from the Unit's water quality flow cell) or biofilm collection insert slides into a bore:

a. Installing flow cells: Select a system tap in the bore pumping system's discharge. Use a dedicated hose to connect to the flow cell. Include a shut off valve and check valve in the inlet system. Mount the flow cell so that outflow is above the inlet and keeps the flow cell full of water. Adjust flow to a trickle, and direct discharge flow to a drain or elsewhere where it does not cause practical problems. Retrieve in about one to two weeks.

b. Retrieving flow cells: The entire flow cell may be removed from the bore, sealing at both ends and keeping it full of water for transport. Alternatively, remove inserts and place in a large zip-lock bag with water to keep the system moist. Return to the lab for analysis.

c. Slide insert in a bore: Secure slides in an insert device, affix securely to nylon line and suspend in the bore below the static or pumping water level (if pumped) or some other depth if desired. Secure the top end of the line to a surface object. Allow slides to collect biofilm for approximately two weeks (may be more or less depending on conditions). Retrieve carefully from the bore, avoiding a hang-up that could break the line. Place the entire collector insert into a zip lock bag with water and return to the lab for analysis.

Sample and Inoculated Tube Disposal:

Inoculated and incubated cultures contain large numbers of bacteria and should be disposed of in a sanitary manner. Droycon Bioconcepts Inc. provides instructions on several methods, including autoclaving (preferred), microwaving in a dedicated (no food) microwave, or immersion in a chlorine solution. After treatment, tubes may be bagged in a dust bin liner and discarded in sanitary trash. PathoScreen tubes may be likewise treated, but bottles can be disinfected and reused. Always wear gloves and avoid splashing when performing these procedures.

Surfaces where samples or tubes have been sitting, and surfaces on and around microscopes when samples are analyzed should be wiped clean and disinfected. Glass slides and cover slips may be cleaned and reused, or discarded in the normal dust bin in the laboratory (following shared laboratory courtesy protocol).

Appendix B: Method illustrations

Biofilm collection methods

Fabricated flowcell collector (Section 9240)

Flowcell in use and exposed slides in insert

Flowcell made from common plumbing parts

”Expedient” flowcell in use (flow from R to L)

Biofilm collector continued

Extracting slides from tubing holders for examination

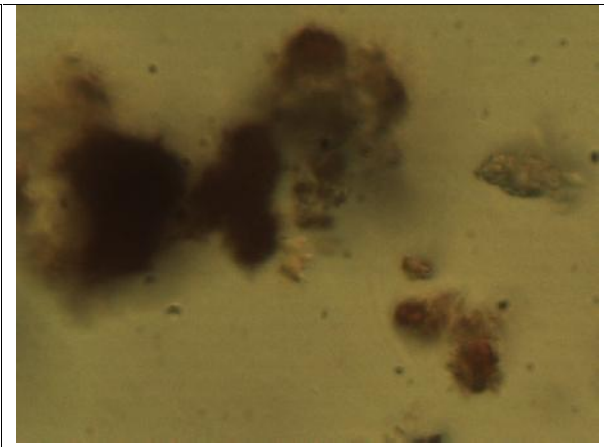


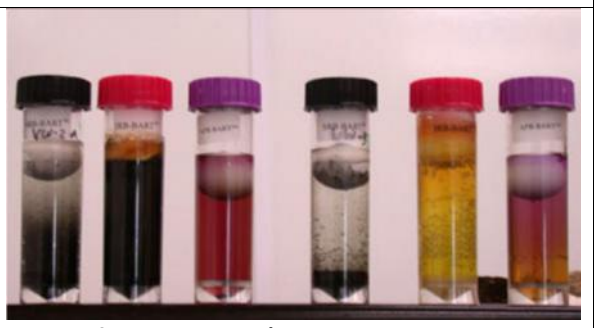
Image from flow cell slide (conventional light microscopy, oil immersion lens in field lab)

Using BART methods in a well evaluation investigation

Inoculating using sterile outer tube – inverted tube center mixing indicator dye. Assembled tube R.

Various reacted BART (negative HAB-BART middle)

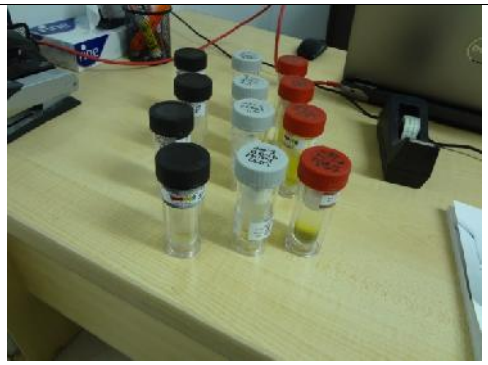
Using BART as field test to evaluate bio contribution of drilling fluid system components (Jordan)



Range of reacted BART (directional wells in alluvial aquifer, New York State, USA) – unreacted APB-BART third from left

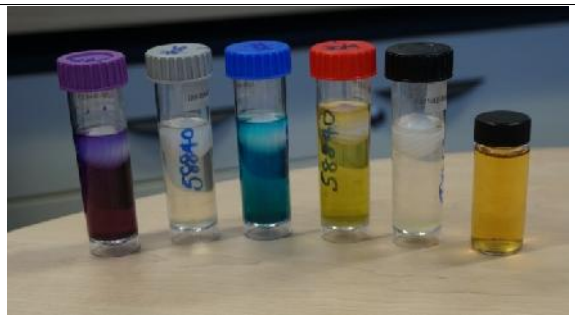
Parallel use of biochemical (qPCR for CENSUS-TRFLP) and BART on same samples

Biofilters ready for shipment for DNA extraction



Range of BART on same water sample

Reacted BART and PathoScreen tubes from an alluvial bore in Queensland



Unresponsive tubes from a deep Precipice Sandstone bore in Queensland with 44 C water.

Example sulfite-reduction packaged medium

CSGCU flow cell unit in use

Sulfur-oxidizing dominant biofouling (Ohio USA, sandstone aquifer)

Nitrate-reducing well biofouling (bacteria related to nonfilamentous S-oxidizing biofilm producers) identified using CENSUS after ambiguous culturing results (Jordan)

PathoScreen™ Field Kit

7. Evaluate the reaction after 24 hours (Table 1). If temperatures have varied significantly, continue to incubate negative samples for an additional 24 hours.

Table 1 Interpreting Results

Hydrogen Sulfide-Producing Bacteria			
Test Results	**Positive**	**Negative**	**Follow-up**
Color changes from yellow to black	X		—
Black precipitate forms	X		—
No color change		X	Incubate additional 12 to 24 hours and reevaluate. If there is no color change, record as negative.

Interpreting the PathoScreen test (from Hach instructions) posted by Engineers without Borders at:

http://my.ewb-usa.org/theme/library/myewb-usa/project-resources/technical/PathoScreen%20Test.pdf (accessed May 2015). Example unreacted and reacted media at right (http://web.mit.edu/watsan/methods_microbiological.html).

BART interpretation charts

Example interpretation chart (they are individual to the test type) from Droycon Bioconcepts. Reaction is interpreted by type (up to three in sequence is useful) and by time up to a limit (in this case, 8 days) individual to the type of test.