Testing for the Presence of Sulfate-Reducing Bacteria

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Experience with testing cooling water and oil field-produced water has shown the common methods of testing for sulfate-reducing bacteria (SRB) can produce misleading results. Various methods of testing for and enumerating SRB are discussed, and the advantages and limitations of each are reviewed. These methods include broth bottles, agar deeps, melt agar tubes, adenosine triphosphate (ATP) assay, a specific (cell surface) antibody method, and a developmental specific antibody technique that tests for an enzyme unique to SRB. These results show no method is perfect, and the limitations of a particular method must be considered in order to obtain meaningful results.

Introduction

Sulfate-reducing bacteria (SRB) are among the most destructive environmental organisms, and their industrial impact is widespread. They cause corrosion and stress corrosion cracking of metals and alloys used in petroleum production and refining,[1-5] cooling water systems,[6-9] waste treatment systems,[10] pulp and paper production,[11] and, in short, all aqueous processes.[12] It was recognized as early as 1910 that SRB are responsible for soil-side pitting corrosion of buried pipelines.[13-17]

SRB produce hydrogen sulfide, which is more lethal than hydrogen cyanide. The sulfide is sometimes used by other organisms to manufacture sulfuric acid,[18] which, in turn, is rapidly destroying concrete municipal sewage systems. Infestation of waterways by SRB[19,20] (the Chesapeake Bay is a recent case in point) is becoming a serious problem and is both a symptom of oxygen depletion and a problem in itself because of the toxic hydrogen sulfide being generated. The combined effect suppresses marine life in the affected area.

The symptoms of these SRB-related problems are obvious: hydrogen sulfide odor, blackening of waters, and black sulfide-corrosion products. By the time these symptoms appear, however, it may be difficult to deal with the problem. Effective treatment is much easier if the infestation is detected at an early stage.

Until recently, microbiological assaying for SRB was limited to culturing the sample in a laboratory until the numbers of SRB were sufficiently high to be detected by crude methods, such as observation of general blackening of the sample. However, such methods have serious limitations,[21,22] resulting partly from the fact that SRB, so pervasive in the environment, are not represented by a single strain or even a single genus but include a diverse collection of different organism types. The types of SRB known to date have differing requirements in regard to both nutrients and temperature.[19,23-26] For this reason, attempts to test for SRB based on growth on one particular medium or at one temperature will always be selective for certain strains.[21,27]

Newer methods propose to speed up this culturing process or to bypass culturing altogether and monitor the organisms directly.[21,28-31] However, physiological differences tend to make the direct methods strain-specific as well.

Testing Methods

This study examines six testing methods. Three of these—broth bottles, agar deeps, and melt agar tubes—are culturing methods. The others—ATP assay, epifluorescence/cell surface antibody, and APS reductase antibody, involve direct detection of SRB.

Initially, the study also employed plate counting as a referee for culturing methods. In practice, however, anaerobic plating proves to be no more reliable than the methods it was intended to referee. This method grew only a few strains of SRB well and was unreliable for other strains. It was therefore eliminated from the study.

Culturing Methods

Researchers have developed countless growth media for use in particular cases.[19,24,32,33] The API RP-38 broth medium was chosen because it is the most widely used culturing method.[34] Two agar methods were also included because they offer shorter testing time. All cultures in this study were incubated at 350 C.

API RP-38 Broth Bottles

Developed by the American Petroleum Institute, and described in its Recommended Practice No. 38 (Alternative Technique for Estimating Sulfate-Reducing Bacteria)[34], this method uses a medium based on lactate.

When SRB are present in the sample, they reduce the sulfate in the medium to sulfide, which reacts with iron in the solution to produce black ferrous sulfide. The blackening of the medium over a 28-day period signals the presence of SRB.

The exact procedure, as spelled out in RP-38, was followed. Briefly, this consists of drawing 1 mL of sample into a syringe and injecting it into a bottle containing 9 mL of liquid growth medium. The bottle is then shaken thoroughly, and a new syringe is used to draw out 1 mL of sample and inject it into a new medium bottle. This procedure is repeated to produce as many serial dilutions as required. (RP-38 specifies a five-bottle series, but as many as 10 or 12 bottles may be necessary when counts are very high.)

It is assumed only a single growing bacterium is required to blacken a bottle. The simplest interpretation of test results, then, is to consider that if one bottle is blackened, the sample contained at least one organism; if two bottles are blackened, the sample contained at least 10 organisms; three bottles blackened, 100 organisms; and so on. Results are usually reported, therefore, as a range; for example, four bottles blackened would be interpreted as 10^3–10^4 cells/ml in the original sample. Where the "≥" symbol appears, it means that all bottles in a series turned black. For example, if six bottles were inoculated and all six turned black, the result would be reported as $\geq 10^5$–10^6.

In cases where duplicate sets of bottles differed by one in the number of bottles turning black, the single value possible in both cases is reported. For example, if in one set of bottles three bottles turned black, and in a duplicate set, four turned black, the numbers inferred are 10^2–10^3 in one case and 10^3–10^4 in the other. For simplicity, only the single overlapping value (10^3) is reported.

On occasion, one bottle in a set will remain clear while bottles at higher and lower dilutions turn black. When this happened, results were interpreted as though the clear bottle had turned black; that is, the last bottle in a series that actually turned black determined the test results.

Agar Deeps

The second culturing technique evaluated is the agar deep, developed by Biosan Laboratories.[31] The medium, described in the appendix, is a slight modification of the RP-38 medium. However, sodium sulfite is included as a reducing agent/oxygen scavenger.

As with the RP-38 bottles, blackening of the medium is the positive test for SRB. Relative numbers of bacteria are estimated in this case by noting the rapidity with which blackening occurs.

The medium is inoculated by dipping a pipe cleaner into the undiluted sample and inserting it into a single vial of semisolid agar. Mineral oil and a CO_2-generating tablet are then added to exclude air, and the vial is capped. It is incubated for up to five days and is checked daily for blackening.

Melt Agar Tubes

This method, developed by the Nalco Chemical Co., uses tryptone as the sole nutrient. Like the agar deeps, it contains sodium sulfite as an oxygen scavenger.

The test procedure involves placing the tubes in boiling water to liquify the medium and then cooling the tubes until the medium reaches a temperature of 40 to 45 C before adding the sample. Again, dilutions of the sample up to 10^6 may be made. These dilutions are pipetted into the bottoms of the tubes. The tubes are then capped tightly and incubated for three days. Results are interpreted by multiplying the number of countable, discrete colonies by the dilution factor to estimate the number of cells/mL of sample. If a tube has general blackening and no visible colonies, SRB were considered too numerous to count.

In theory, if one or more tubes show general blackening, then a subsequent tube should have a countable number of discrete colonies. Where this was not the case, the last blackened tube was considered in this study to have >100 colonies. For example, if a series of four tubes was inoculated and the first two turned black but the other two had no visible colonies, the result would be reported as $>10^3$.

Direct Methods

Unlike culturing techniques, direct methods require no SRB growth during the test. Rather, they

test directly for the presence of SRB cells or for compounds present in SRB cells. Three direct methods were studied.

ATP Assay

This method estimates the total number of organisms by measuring the amount of adenosine triphosphate (ATP) in a sample. ATP is a compound found in all living matter. Littmann has proposed that ATP assay techniques may be used with oil field water samples to estimate relative numbers of organisms including SRB.[35,36] This method has not been proposed, nor was it evaluated in this study, for other field sample uses relative to SRB.

The procedure involves filtering a sample to remove dissolved solids and salts that might interfere with the test.[37] This filtered sample is then added to a reagent that releases the cells' ATP. A special enzyme that reacts with ATP to trigger a photochemical reaction is added, and the resulting emitted light is measured by a photometer. The number of bacteria cells is estimated from the total light counted.

Internal standards are also run and standard curves plotted to ensure that the instrument and the reagents are working properly and that any remaining interferences in the sample are not giving false readings. While reagents and photometers are available from more than one source, a Science Applications Inc. Model 3000 integrated photometer was used in this study, and reagents were obtained from Turner Designs Co.

Epifluorence/Cell Surface Antibody

This method, referred to in this paper as the ECSA method, is based on specific antibody attachment to SRB cells.[28] Antibodies, tagged with a fluorescent compound, attach only to specific sites on the surface of SRB. When viewed under an epifluorescence microscope, an antibody-linked cell is identified by a green fluorescent border.

The procedure involves heat-fixing the sample to a special slide and then incubating it for 20 min. with a primary antibody reagent. After the sample is washed to remove excess antibody, it is then incubated for 20 min. with a second antibody reagent containing the fluorescent compound. The sample is then rewashed, and a special mounting fluid and cover slip are added. Counting is done at 1000X magnification under oil immersion.

This method was developed by D. H. Pope at the Rensselaer Polytechnic Institute, under contract to the Materials Technology Institute of the Chemical Process Industries (MTI). Necessary reagents were obtained from Bioindustrial Technologies Inc.

Pope acknowledges that, while this method appears to work well on field samples, it does not consistently work well on laboratory-cultured SRB. He believed the antigenic properties of the cell surfaces change after repeated laboratory culturing.

APS Reductase Antibody

This method, abbreviated herein as the ARA method, was under development by the Du Pont Co. at the time of this study. It involves antibodies developed against adenosine-5'-phosphosulfate (APS) reductase, an internal enzyme found in all SRB.[38] The sample is first washed to remove interfering chemicals such as hydrogen sulfide. It is then treated ultrasonically in a small, battery-powered instrument that breaks open the bacteria cells and releases the APS reductase enzyme.

The remainder of the test takes place within a polyethylene transfer pipette. The sample is drawn into the pipette past a treated porous bead. It is washed four times, and then a color developing solution is drawn through the bead. If the APS reductase enzyme is present in the sample (indicating the presence of SRB), the bead turns blue within 10 min. The degree of coloration is proportional to the amount of the enzyme in the sample, and results are read on a color chart supplied with the kit. The chart allows the user to correlate the color obtained with approximate numbers of SRB.

Evaluation Criteria

The various methods were evaluated on the basis of six criteria: accuracy, specificity, ease of use, time required to obtain results, appropriateness for lab or field use, and obvious drawbacks and limitations. Each criteria is discussed below.

Accuracy

To determine relative accuracy, samples containing known numbers of SRB cells were tested by each method in the laboratory. These samples consisted of pure cultures of three different strains of *Desulfovibrio desulfuricans*.

Specificity

Whereas accuracy implies the ability to count total SRB numbers, specificity relates to the ability to (1) count all various strains of sulfate reducers, and (2) discount other bacteria types. For laboratory research, it may be desirable for a method to be very specific for one strain of SRB; for general field use, however, ability to detect total SRB numbers is usually more desirable. (To date, no evidence exists that any one SRB strain is more destructive than another.)

Ease of Use

Some methods require a high level of training or skill for successful use, while others can be used with little training by relatively unskilled people. While these factors may not affect the ability to get

good test results, they do affect the practicality of having SRB numbers checked frequently in the field.

Time Required to Obtain Results

The value of this criterion depends on the circumstances under which testing occurs. If a sample is being tested as part of a failure analysis (that is, to confirm that SRB are present at a corrosion site), time required to obtain results may not be important. On the other hand, if testing is designed to determine whether biocide should be added to a dynamic system (for example, an oil field water injection system), results are most useful if obtained in a matter of minutes; results obtained in one to four weeks are probably of little value.

Field vs Laboratory Use

The ability to run tests in the field offers several obvious advantages. Some tests can be performed adequately at the sampling site, while others require special conditions or equipment found only in a laboratory. The ideal method would be performed with equal confidence in either field work or in a laboratory.

Obvious Drawbacks and Limitations

This criterion covers all perceived negative aspects of each method. These negatives should be evaluated on a case-by-case basis. The inability to test very dirty samples of cooling water or sludge, for example, may not be a problem when testing oil field water samples, which are usually relatively clean except for traces of oil.

Test Results

Testing in this study falls into two broad categories: (1) tests on pure SRB strains of known cell concentrations and (2) tests on field samples of unknown bacterial composition. The first category is useful for determining the relative accuracy and specificity of the methods. Field samples, on the other hand, test how well each method can handle solids and chemical interferences.

Samples of Known Composition

Desulfovibrio desulfuricans G100A

A pure culture, isolated from an oil-field-produced water, was suspended in a water base produced by autoclaving a mixture of cooling water samples. The cells in this suspension were quantified in a Petroff-Hauser counter using a phase-contrast microscope. The cell suspension was then sparged with nitrogen gas to remove residual hydrogen sulfide and diluted with varying amounts of the water base to produce samples with known concentrations of G100A as high as 3×10^8 cells/mL. Results are shown in Table 1.

TABLE 1
Test Response vs Known Numbers: G100A Strain

Actual Count	SRB Numbers Obtained With: RP-38	Agar Deeps	Melt Agar	ATP	ECSA
0	Neg	Neg	Neg	8×10^4	Neg
3×10^2	$\geq 10^3 - 10^4$	$>10^2$	70	5×10^4	Neg
3×10^4	$\geq 10^4 - 10^5$	$\geq 10^3$	$>10^2$	7×10^4	Neg
3×10^6	$\geq 10^6 - 10^7$	$\geq 10^5$	Neg	3×10^5	3×10^5
3×10^8	$\geq 10^8 - 10^9$	$\geq 10^5$	60	8×10^6	10^6

The G100A strain was being supported on a lactate medium. The physiology of this strain has been reported by Weimer et al.[39]

Among culturing methods, the RP-38 broth bottles gave accurate numbers at higher cell concentrations but overestimated numbers at low cell concentrations. The agar deeps, on the other hand, performed well at low cell concentrations but greatly underestimated high cell concentrations. The melt agar tubes performed poorly in this test.

The ECSA method did not detect numbers below 10^6 cells/mL, and both that method and the ATP assay underestimated by one or two orders of magnitude above that level.

The ARA method is not included in Table 1 because the method was derived from the G100A strain and calibrated in this experiment. The response curve is shown as Figure 1 and is plotted as known numbers of cells versus color "number" as determined by direct comparison with a cyan-blue step chart. A reading of 1 or 2 is white (that is,

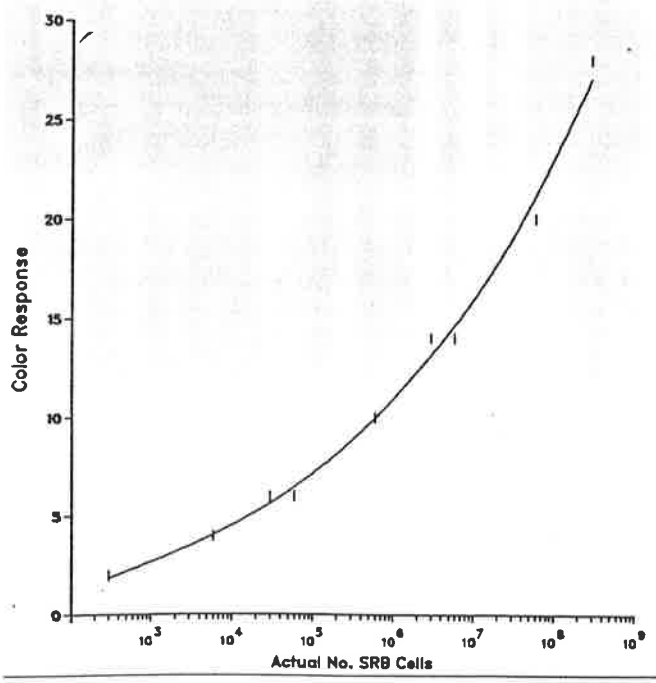

FIGURE 1
ARA test response vs known numbers.

SRB too few to count), and a reading of 32 is dark cyan. All other experiments described in this paper report results obtained with the ARA method as "equivalent G100A" numbers extrapolated from this calibration curve for the method.

Desulfovibrio desulfuricans Norway Strain.

A pure culture obtained from H. D. Peck Jr. (University of Georgia) was transferred in the laboratory to a lactate medium and incubated overnight at 30 C to a cell concentration of approximately 10^9 cells/mL. The cells were spun down at 15,000 rpm, the culture medium was decanted off, and the cells were resuspended in 50 mM Hepes buffer. Cells were counted as with the G100A strain, and dilutions were made in deaerated Hepes buffer to achieve cell concentrations ranging from 10^3 to 10^8 cells/mL for testing. Results are shown in Table 2.

Blackening of the RP-38 broth bottles did not begin until about five days incubation had elapsed at 35 C. This slow growth at 35 C, compared to the rapid growth observed in the original culture at 30 C, implies the optimum temperature for this particular strain is well below the 35 C level selected for this study, which emphasized the limitation inherent in selecting any one incubation temperature for testing samples of unknown biological composition.

RP-38 broth bottles underestimated numbers by several orders of magnitude. These numbers might have been higher if the test had continued past 28 days, again a reflection of the slow growth observed. The two agar methods showed essentially no growth within the test periods specified for those methods, although significant blackening was observed later. This result demonstrates the risk associated with "rapid" culturing methods.

Like the RP-3S broth bottles, the ATP assay and the ARA methods both underestimated numbers, while the ECSA method could not detect any of these cells.

In summary, none of the methods performed well with the Norway strain, especially at cell concentrations below 10^6 cells/mL.

Desulfovibrio desulfuricans API Strain

A pure culture of Mid-Continent Strain A was obtained from Conoco Inc., as recommended in API RP-38. The culture was transferred in the laboratory and regrown to a cell density of about 2×10^9. These growing cells were spun down at 15,000 rpm and resuspended in 50 mM Hepes buffer. Cells were quantified as with the previous tests and diluted into deaerated Hepes buffer to cell concentrations ranging from 10^3 to 10^8 cells/mL. Results of tests on these samples are shown in Table 3 on page 76.

The RP-38 method showed excellent correlation with known numbers, which is not surprising, because the method was developed using this strain

TABLE 2
Test Response vs Known Numbers: Norway Strain

Actual	SRB Numbers Obtained With:					
Count	RP-38	Agar Deeps	Melt Agar	ATP	ECSA	ARA
10^8	$10^4 - 10^5$	Neg	10	1×10^7	Neg	4×10^6
10^7	$10^3 - 10^4$	Neg	Neg	8×10^5	Neg	9×10^4
10^6	$10^2 - 10^3$	Neg	Neg	6×10^4	Neg	4×10^4
10^5	$10 - 10^2$	Neg	Neg	5×10^4	Neg	6×10^2
10^4	1 – 10	Neg	Neg	7×10^3	Neg	6×10^2
10^3	Neg	Neg	Neg	1×10^4	Neg	Neg
10^2	Neg	Neg	Neg	8×10^3	Neg	Neg
0	Neg	Neg	Neg	1×10^4	Neg	Neg

of SRB. The agar deeps were considerably less sensitive and less accurate. In addition, duplicate tests gave widely inconsistent results in some cases. The melt agar tubes were even less reliable, indicating about five orders below actual numbers.

ATP assay, as it did with other pure strains, underestimated cell numbers by two to three orders of magnitude. The ECSA method again failed to detect a pure laboratory strain. The ARA method was generally accurate above 10^4 cells/mL, but it did not detect lower cell concentrations.

In summary, the RP-38 method was most accurate in counting low numbers of the API strain. That method and the ARA direct method both gave good results above about 10^4 cells/mL.

Sulfite Reducers

As mentioned earlier, only SRB are capable of reducing sulfate. Several other common environmental bacteria, however, are capable of reducing sulfite to sulfide.[40] Two of the culturing media tested contain sulfite as a reducing agent/oxygens Scavenger. To determine the extent to which this sulfite might give false positive results if such organisms are present, sterile buffer solution was spiked with each of four different bacterial strains believed to contain the enzyme capable of reducing sulfite to sulfide (sulfite reductase): *Escherichia coli*, *Edwardsiella tarda*, *Proteus mirabilis*, and *Citrobacter freundii*. The results are summarized in Table 4 on page 77. These cultures, like the Norway strain, were obtained from H. D. Peck Jr. The results showed that all organisms tested, except *E. coli*, gave false positive results when using the two sulfite-containing media.

Field Samples of Unknown Bacterial Composition

Oil Field Water Samples

Thirteen water samples from oil production in Oklahoma and Texas were tested. These represented a variety of sources: injection water from waterflood fields, produced water from those fields

TABLE 3
Test Response vs Known Numbers: API Strain

Actual Count	SRB Numbers Obtained With: RP-38	Agar Deeps	Melt Agar	ATP	ECSA	ARA
0	Neg	$>10^5$, Neg	Neg	4×10^3	Neg	Neg
10^2	10	Neg	Neg	4×10^3	Neg	Neg
10^3	10^2	Neg	Neg	3×10^3	Neg	Neg
10^4	$10^3 - 10^4$	$\geq 10^4$	17, $>10^3$	7×10^3	Neg	2×10^3
10^5	$10^4 - 10^5$	$>10^4$, Neg	Neg	2×10^4	Neg	2×10^5
10^6	$10^5 - 10^6$	$>10^5$, Neg	80,25	2×10^4	Neg	9×10^6
10^7	10^7	$>10^5$	3.5×10^2	9×10^4	2×10^4, Neg	2×10^7
10^8	$10^7 - 10^8$	$>10^6$	2×10^3	9×10^5	4×10^4, Neg	5×10^7

Double values separated by commas indicate duplicate tests gave widely differing results.

(drawn directly from wellheads), and separated water phase from knockout drums. Results are shown in Table 5 on page 77.

Among the culturing methods, results from the agar deeps and RP-38 broth bottles correlated fairly well, with two notable exceptions: sample numbers 8 and 12. In the case of No. 8, the agar deeps failed to detect SRB while the RP-38 bottles (as well as the direct ARA method) indicated significant numbers. The reverse is true with No. 12, in which the agar deeps indicated very high numbers while all other methods reported low numbers. The melt agar tubes were not used on sample numbers 1 through 8, but this method showed low numbers relative to the other methods on the other five samples.

This set of samples was the most important test of the ATP assay, because its use for detecting SRB is proposed only for oil field water samples. It showed reasonable correlation with RP-38 numbers on only four of twelve samples and with the ARA method on only three of twelve samples. It is this inconsistency, more than the absolute numbers, that is disturbing. Many types of microorganisms other than SRB grow in oil field waters, and this method is incapable of distinguishing among them. It is assumed that the non-SRB organisms varied widely in these samples and accounted for the inconsistent results with the ATP method.

The ECSA method, as proposed, was able to detect cells in field samples better than in laboratory samples. Of the five samples tried with this method, one contained too much debris to permit counting of cells.

The ARA method compared reasonably well with RP-38 broth bottles when cell numbers were above 10^3 cells/mL, except in the case of sample No. 8, when the ARA method reported much higher numbers. At lower cell concentrations, results from the two methods often did not correlate.

Cooling Tower Sludge Samples

Three sludge samples from the bottoms of cooling towers were tested. While it was suspected that all three would contain countable SRB, the main purpose of these samples was to test the ability of various methods to handle "high-solids" samples. In addition to SRB, cooling water sludge samples would normally be expected to contain a rich assortment of bacteria—especially anaerobic and facultative anaerobic strains.[7] Results are summarized in Table 6 on page 78, samples CS1-CS3.

The high-solids samples created problems with all methods: samples could not be drawn into pipettes or syringes until after they were first put through a small kitchen strainer to remove sand and large clumps; the black sludge coating the pipe cleaner in the agar deep test made it difficult to judge when blackening by sulfide generation was occurring; the ATP test could not be performed at all because the sample was not filterable; particles adhering to the ECSA slide made that test almost

TABLE 4
Sulfite Reduction Tests

Organism Strain	Actual Count	SRB Numbers Obtained With: Agar Deeps	Melt Agar
E. coli	10^6	Neg	Neg
E. tarda	10^6	$\geq 10^4$	$>10^3$
P. mirabilis	10^6	$\geq 10^2$	$>10^3$
C. freunidii	10^6	≥ 10	$>10^3$

impossible to interpret. The ARA test was run with relative ease using a proprietary filtering procedure offered with the method.

Cooling Water Samples

Six different cooling water samples representing a variety of open and closed systems were analyzed. Results are summarized in Table 6, samples CW1-CW6. Unlike the cooling tower sludge samples, the cooling water samples showed wide discrepancies among the various methods. The two specific antibody methods (ECSA and ARA) showed generally higher numbers than did the RP-38 bottles, which could imply high counts of SRB that do not grow on lactate (an implication confirmed by independent study of one system represented here).

Waste Treatment Samples

Six samples were drawn from aerobic (biological) waste treatment systems at two different plant sites. While most of these samples had a much lower level of solids content than seen in the cooling tower sludge samples discussed above, they would be considered "dirty water." None of these samples required screening through a kitchen strainer, but most proved difficult to draw into pipettes or syringes.

Results are summarized in Table 7 on page 78.

General Evaluation

Culturing Methods

Before beginning an evaluation of the three culturing methods, it is important to make some observations regarding the advantages and disadvantages common to all such methods.

The distinct advantage of culturing techniques is that they are uniquely sensitive because they allow low numbers of SRB to grow to easily detectable higher numbers. However, culturing methods also have significant disadvantages that can lead to underestimation of numbers:

1. Growth media tend to be strain-specific. For example, lactate-based media will grow lactate oxidizers but not acetate-oxidizing strains. Attempts to produce useful "all-purpose" SRB media have apparently been unsuccessful.
2. Incubating at any one temperature is selective for strains that will grow at that temperature.
3. SRB may die or grow slowly when removed from their preferred environment. This is especially true if the sample is exposed to air.
4. Culturing methods cannot distinguish between a single SRB cell and a clump of SRB cells.

For these reasons, culturing methods are likely to underestimate true numbers.

TABLE 5
Test Response on Oilfield Water Samples

Sample Number	SRB Numbers Obtained With: RP-38	Agar Deeps	Melt Agar	ATP	ECSA	ARA
1	$\geq 10^4 - 10^5$	$\geq 10^5$	—	2×10^4	—	9×10^5
2	Neg	Neg	—	BDL[1]	—	Neg
3	$10 - 10^2$	Neg	—	10^6	—	10^4
4	$1 - 10$	Neg	—	3×10^5	—	3×10^3
5	$1 - 10$	Neg	—	9×10^4	—	Neg
6	Neg	Neg	—	7×10^4	—	3×10^3
7	$10^2 - 10^3$	≥ 10	—	3×10^4	—	Neg
8	$10^3 - 10^4$	Neg	—	—	—	10^7
9	10^3	$\geq 10^5$	3×10^2	2×10^6	Neg[2]	4×10^3
10	$\geq 10^4 - 10^5$	$\geq 10^6$	2×10^2	2×10^5	5×10^4	4×10^4
11	$\geq 10^4 - 10^5$	$\geq 10^5$	6.5×10^2	9×10^5	2×10^4	6×10^2
12	10^2	$\geq 10^6$	6	3×10^4	5×10^4	6×10^2
13	10^3	$\geq 10^4$	Neg	2×10^6	Neg	Neg

[1]Below detectable limit.
[2]Too much debris to see any cells.

TABLE 6
Test Response on Cooling Tower Sludge and Water

Sample Number	SRB Numbers Obtained With: RP-38	Agar Deeps	Melt Agar	ECSA	ARA
CS1	$10^3 - 10^4$	$>10^6$	$>10^3$	5×10^6	2×10^5
CS2	$\geq 10^5 - 10^6$	$>10^6$	$>10^3$	0[1]	2×10^5
CS3	$\geq 10^5 - 10^6$	$>10^4$	$>10^3$	0[1]	3×10^4
CW1	$10 - 10^2$	Neg	Neg	2×10^6	3×10^4
CW2	Neg	Neg	Neg	–	Neg
CW3	$10 - 10^2$	$>10^4$	Neg	–	10^4
CW4	$10 - 10^2$	$>10^5$	$>10^3$	10^6	2×10^5
CW5	Neg	Neg	$>10^2$	–	3×10^4
CW6	$10 - 10^2$	$\geq 10^5$	Neg	–	5×10^4

[1]Too much debris to see any cells.

TABLE 7
Test Response on Waste Treatment Water and Sludge

Sample Number	SRB Numbers Obtained With: RP-38	Agar Deeps	Melt Agar	ECSA	ARA
WT1	$\geq 10^5 - 10^6$	$>10^6$	$>10^3$	3×10^6	3×10^5
WT2	$\geq 10^5 - 10^6$	$>10^6$	$>10^3$	5×10^6	4×10^5
WT3	$\geq 10^3 - 10^4$	$>10^6$	$>10^2$	–	3×10^4
WT4	$\geq 10^5 - 10^6$	$>10^5$	$>10^3$	3×10^6	2×10^4
WT5	$10^3 - 10^4$	$>10^6$	$>10^2$	10^5	2×10^5

In addition to these general statements about culturing techniques, comments specific to each method follow. Advantages are preceded by a "+," and disadvantages are noted by a "–."

RP-38 Broth Bottle

+ This method was the most consistently accurate of the three culturing techniques evaluated.

– Four weeks are required for final results. (Some people interpret after shorter times, but it was found that 28-day results sometimes differ significantly from 14- or 21-day results.)

– This method requires the use of "sensitive" equipment, namely hypodermic needles and media bottles, which are difficult to transport on airplanes and across state lines because of their association with drug abuse.

Agar Deeps

+ The agar deep is the easiest of these methods to use.

+ No special or "sensitive" equipment is needed.

+ It is easy to wipe surfaces with the pipe cleaner and thus sample biofilms for testing.

+ Dirty or high-solids samples can be handled easily with this method because samples do not have to be forced through a filter or a needle. On the other hand, darkening of the pipe cleaner by very dirty samples can mask early blackening due to SRB sulfide production.

± Results are determined in a maximum of five days. On the positive side, this allows the user to act faster if SRB numbers start to increase. On the other hand, five days is often insufficient time for SRB to become acclimated to the medium and grow. Many cases were observed in which blackening occurred only after the five-day test period had elapsed.

– It may be inconvenient to read the test every day over the five-day incubation period because this period always includes at least part of a weekend.

– The test may yield false positives because the medium contains sulfite, which certain common non-SRB can reduce to sulfide.

Melt Agar Tubes

+ No special or "sensitive" equipment is needed.

± Test results are obtained within three days. This period involves the same positives and negatives given for the agar-deeps.

– The procedure involves placing the tubes in boiling water to melt the medium and then cooling them to 45 C, a process difficult to control in the field.

– The serial dilutions employed by this method are tedious and complicated compared to those required for the RP-38 bottles.

– Results are difficult to interpret. The directions specify that spherical colonies must be observed, yet, in many cases, the entire tube turns black so that individual colonies cannot be discerned.

– This medium, as with the agar deeps, contains sulfite and can, therefore, gives a false positive.

Direct Methods

Direct methods, in general, have two distinct advantages over culturing methods: first, they give rapid results because they do not require an incubation period; second, because they do not require that SRB grow, they avoid the underestimation of numbers associated with culturing methods.

On the negative side, however, direct methods are relatively insensitive in that 10^3 or more cells are needed for detection.

Sensitivity can be improved in some cases by using filtration or centrifugation to concentrate the sample.

Direct methods have another disadvantage: they all involve biological reagents, which require refrigerated storage to assure reasonable shelf life.

Comments specific to each method follow. Again, advantages are preceeded by a "+" and disadvantages are noted by a "–".

ATP Assay

+ Results can be obtained in less than one hour.

Because this method monitors the amount of ATP in the sample, it is not strain-specific: it can detect the ATP in all strains of SRB. At the same time, however, it will also detect the ATP in other types of bacteria that might be in the sample, so it has a poor specificity for SRB.

– The method is suitable for use only on relatively clean samples. Sample preparation involves filtration to remove substances that may interfere with the test, and dirty, high-solids samples are not suitable for filtration.

– The equipment used in this method is fairly expensive. A typical photometer costs $5,000 to $10,000.

ECSA (Epifluorescence/Cell Surface Antibody

+ Results can be obtained in two to three hours.

+ The method is specific for SRB, yet relatively nonstrain-specific among SRB.

– The detection threshold for this method is 10^4 cells/mL.

– The test is sometimes difficult to interpret, especially with dirty samples in which fluorescing debris obscures the bacteria. This method requires a high level of training to obtain accurate results.

– This method uses an expensive epifluorescence microscope, which typically costs $10,000 to $25,000.

APS Reductase Antibody

+ Results are obtained in about 15 min.

+ Results of the test are easy to interpret. Color reactions indicate positive tests; no color indicates a negative test.

+ The method is specific for SRB.

+ No expensive or "sensitive" equipment is needed to perform the test.

+ This method is the only one that allows field interpretation and does not involve use of a laboratory facility.

– The detection threshold for this method is 10^3 cells/mL.

Conclusions

1. The culturing methods, in general, were able to detect lower numbers of SRB than the direct methods, although they are somewhat strain-specific.

2. API RP-38 broth bottles appear to offer the best combination of sensitivity and accuracy when working with lactate-utilizing organisms such as *Desulfovibrio sp.* The best practice for accurate results with the bottles is to allow 28 days for incubation (as specified in RP-38) rather than 14 or 21 days as is common practice.

3. Agar deeps were the easiest of the culturing methods to inoculate in the field and offer results in less time than the RP-38 broth bottles. Nonetheless, they frequently did not correlate well with other methods and proved to give false positives with sulfite-reducing bacteria. For these reasons, use of agar deeps is recommended only if this method is first validated in any given system by correlating numbers obtained with agar deeps to numbers obtained with RP-38 broth bottles.

4. The melt agar tubes appeared insensitive to low numbers and greatly underestimated high numbers of SRB. This result, coupled with their tendency to give false positives in the presence of non-SRB sulfite reducers, renders them poorly suited for monitoring SRB.

5. ATP assay results did not correlate with SRB numbers. It overestimated SRB numbers in field samples and underestimated numbers in pure SRB samples. ATP assay is probably useful only in cases where a total bacterial count is desired.

6. The ECSA (epifluorescence/cell surface antibody) technique appears to be a valid method for detection of SRB in field water samples, with the stipulation that it is accurate only when used by highly trained personnel. Its lower limit of detection in field samples is approximately 10^4 cells/mL.

The ECSA method is not valid for detecting laboratory strains of SRB and is inappropriate for testing field samples with large quantities of suspended solids.

7. The ARA (APS reductase antibody) method is the fastest method and the only true field technique of those tested. Results obtained with this method were generally comparable to those obtained with RP-38 broth bottles. At its present developmental stage, however, the ARA method has a detection threshold of about 10^3 cells/mL.

8. No one method was superior in all respects. For this reason, it would seem prudent to use the above methods in combination to obtain best results.

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