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API
RECOMMENDED PRACTICE
For
BIOLOGICAL ANALYSIS OF SUBSURFACE INJECTION WATERS

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NOTE

The second edition, December 1965, superseded the first edition, May 1959, titled *Recommended Practice for Biological Analysis of Water-flood Injection Waters*. This third edition supersedes the second edition.

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API RECOMMENDED PRACTICE FOR BIOLOGICAL ANALYSIS OF SUBSURFACE INJECTION WATERS

FOREWORD

a. This recommended practice has been developed by the Subcommittee on Biological Analysis of Water to be Used for Water-flood Purposes, of the Committees on Drilling and Production Pactice, in cooperation with research laboratories of operating and chemical companies and university life sciences departments. Membership of the committee is listed on the preceding page.

b. The objective of the study committee was to set up standard methods of tests for determination of the effectiveness of chemicals for treating injection waters to control the growth of microorganisms and to develop procedures for the biological analysis of injection waters. It is believed that these methods will help eliminate some of the confusion concerning biological analysis of injection waters. The use of the standard procedures will allow comparison of data from various water floods.

SECTION I

EXAMINATION FOR MICROORGANISMS

Scope

1. A knowledge of the microbial population of an injection water is necessary to determine whether a biological problem exists. This knowledge is also necessary to evaluate the effectiveness of a chemical treatment.

2. These procedures are intended to reveal the number and types of microorganisms present in a particular water sample.

Purity of Reagents

3. Unless otherwise indicated, all chemical reagents shall meet American Chemical Society* specifications. Prepared bacteriological media can be obtained from biological supply houses.

Sampling Methods

4. The following sampling techniques should be adhered to:

a. A clean sterile bottle will be used.

b. The tap should be allowed to flow at least 3 min at the sampling rate prior to taking the sample.

c. The sample should be taken in such a manner as to preclude contamination from external sources.

d. The time, date, temperature, and appearance of the water should be recorded at the time of sampling, and this information should be included with the sample. If possible, the total solids and/or mineral content of the water should be included.

e. The sample should be handled in such a manner as to avoid radical changes in temperature between time of sampling and time for examination of the sample.

f. The maximum time between sampling and examination should not exceed 24 hours. The sample should be cultured as soon as possible. If an examination cannot be initiated within the 24-hour period, the following statement should be included in the report: "These results do not necessarily represent the actual microbial content of the water at the time of sampling."

g. Slime masses and other deposits should be sampled separately.

Microscopic Examination of Water

5. Microscopic examination makes possible the detection of microorganisms which are not easily cultivated on laboratory media. In addition, it may permit an estimate of the microbial population. This information may be used as a guide in making plate counts.

6. The best method of examination is by the use of the phase contact microscope. With this microscope, one can more easily view the microorganisms and avoid the distorting tendencies of some of the staining techniques.

7. The examination should include identification of microorganisms as follows:

a. Algae and protozoa
   (1) Flagellates
   (2) Ciliates
   (3) Diatoms
   (4) Filamentous

b. Bacteria
   (1) Iron bacteria — describe the type, i.e., sheath or stalk
   (2) Sulfur bacteria for the presence of sulfur granules
   (3) Bacterial slime
   (4) Total direct microscopic counts

c. Fungi

8. With the exception of 7.b. (4), the microscopic estimates of each type shall be reported as none, present, present in large numbers.

9. The membrane filter is very useful in the detection and enumeration of iron bacteria, algae, and protozoa. A measured quantity of the water under examination can be filtered through the membrane filter. The filter should be dried, cut into small sections, and the sections placed on microscope slides. The filter sections can be rendered transparent by a drop of immersion oil, then subjected to the usual microscopic examination.

General Bacterial Counts of Injection Waters

10. The following medium* shall be used for making bacterial counts on waters containing less than 20,000 ppm (mg/1 liter) total solids:

Beef extract, grams	3.0
Tryptone, grams	5.0
Dextrose, grams	1.0
Agar, grams	15.0
Distilled water, milliliters	1,000.0

11. Adjust pH to 7.0 with sodium hydroxide (NaOH) and sterilize at 15 lb steam pressure for 15 min.

12. The sulfate-reducing bacteria medium (Par. 18) shall be used for making plate counts on waters containing over 20,000 ppm total solids.

13. Pour plates are more desirable than spread plates because of minimization of surface spreading colonies. Dilutions and plate counts are to be made in the manner prescribed by the latest edition of *Standard Methods for the Examination of Water and Waste Water.*†

14. Plates shall be incubated under aerobic conditions and within 5 C of the recorded temperature of the water when sampled.

15. Plates should be observed for growth after 2 to 5 days' incubation.

16. For untreated waters, bacterial counts of less than 10,000 organisms per milliliter are generally of little significance. The significance of counts above 10,000 per milliliter will depend upon additional evidence such as loss of injectivity, increased wellhead pressure, or filter plugging.

17. A suitable broth culture method for aerobic bacteria is presented as Appendix A, Section A-II, Par. A2.

Sulfate-reducing Bacteria Counts

18. A medium§ of the following composition shall be used for counting sulfate-reducing bacteria:

Sodium lactate, USP, milliliters	4.0
Yeast extract, grams	1.0
Ascorbic acid, grams	0.1
$MgSO_4 \cdot 7H_2O$, grams	0.2
K_2HPO_4 (anhydrous), grams	0.01
$Fe(SO_4)_2(NH_4)_2 \cdot 6H_2O$, grams	0.2
NaCl, grams	10.0
Agar, grams	15.0
Distilled water, milliliters	1,000.0

NOTE: Resazurin, 0.001 grams per liter, may be added as an indicator for the presence of oxygen.

19. The ingredients should be dissolved with gentle heating. The pH should then be adjusted to 7.3 with NaOH. If excessive precipitation occurs, the medium should be discarded. The medium is dispensed into test tubes (9 ml per tube), which are then autoclaved for 10 min at 15 psi steam pressure.

20. After autoclaving, the tubes are cooled to 45 C in a water bath. One milliliter of the water sample is transferred to the first tube. The tube is stoppered and flowed back and forth four times to mix the inoculum. One milliliter from this tube is then transferred to a second tube and mixed as before. Continue this serial transfer until a dilution of 1 to 10,000 is reached (5 tubes). After the inoculum has been transferred from each tube, the tube should be cooled rapidly to solidify the agar. (Screw caps or rubber stoppers should be used to seal the tubes to prevent dehydration of the medium.) To prevent solidification of agar in the transfer pipettes, warm pipettes (near 45 C) should be used for this operation.

21. All work is done in duplicate and tubes are incubated at a temperature within 5 C of the recorded temperature of the water at the time of sampling.

22. All tubes should be held a minimum of 4 weeks. The tubes should be examined on the third day and at the end of each week for the appearance of sulfate-reducing bacteria, as indicated by intense black colonies.

23. The presence of sulfate-reducing bacteria is considered to represent a potential problem. The extent of the problem will depend upon additional evidence, such as black water or an increased hydrogen sulfide content of the injection water.

24. A suitable field test for the culture of sulfate-reducing bacteria is presented as Appendix A, Section A-II, Par. A7-A12.

*This medium may be purchased in the dehydrated form as TGE Agar.

†Available from American Public Health Assn., Inc., 1790 Broadway, N.Y., N.Y. 14212.

§Sulfate-reducing bacteria medium can be obtained in dehydrated form from the biological supply houses.

SECTION II

EVALUATION OF CHEMICALS FOR CONTROL OF MICROBIAL GROWTH

Scope

25. These procedures are intended to be used to evaluate chemicals as anti-microbial control agents in water-injection systems where microbial growth is a problem, and where it is believed chemical control can be expected to yield a desirable end result.

26. These methods are designed to be used for screening purposes in the laboratory. Application to specific microbial-control problems may require modification of these techniques (see Appendix A).

Purity of Reagents

27. Unless otherwise indicated, all chemical reagents shall meet American Chemical Society specifications. Only [redacted] grades of agar and yeast extract shall be used.

Bacteriostatic Test — Sulfate-reducing Bacteria

28. The composition of the sulfate-reducer medium for the bacteriostatic test is as follows:

Sodium lactate, USP, milliliters	4.0
Yeast extract, grams	1.0
Ascorbic acid, grams	0.1
$MgSO_4 \cdot 7H_2O$, grams	0.2
K_2HPO_4 (anhydrous), grams	0.01
$Fe(SO_4)_2(NH_4)_2 \cdot 6H_2O$, grams[1]	0.2
NaCl, grams	10.0
Distilled water, milliliters	1,000.0

[1]Added after sterilization.

29. The ingredients are dissolved by gentle heating with constant stirring. After dissolution has occurred, the pH of the medium should be adjusted to 7.3 with NaOH. If necessary, the medium is then filtered through Whatman No. 1 or No. 2 filter paper. Following filtration, the medium is autoclaved at 15 lb steam pressure for 10 min. After autoclaving, the medium is allowed to cool slightly and the iron salt is added. The pH is then checked and readjusted if necessary. The medium is now cooled to room temperature as rapidly as possible, without agitation.

30. After cooling, the medium is inoculated from the third successive 24-hour transfer of an actively growing culture* of sulfate-reducing bacteria. Ten milliliters of inoculum are added to each liter of medium.

31. From stock solutions of the chemical to be screened, sufficient amounts of the compound should be added to 1- or 2-oz clear bottles so that when the containers are filled, the desired concentrations of chemical are present. The amount of stock solution added should not exceed 10 percent of the volume of the test bottle. After addition of the chemical, the bottles are completely filled with the inoculated medium. The bottles are then capped with plastic caps containing cork and aluminum foil or teflon liners and incubated at 30 C ± 2 C.

32. All work is done in duplicate with at least three controls for each series. The controls consist of bottles filled with inoculated medium only. Growth of sulfate-reducing bacteria in the bottles is indicated by an intense blackening of the medium, whereas containers having sufficient chemical to inhibit growth will remain clear. Bottles with no sulfate-reducing bacteria growth are observed for a period of 28 days after the controls have blackened.

33. If the compound under test is effective at less than 50 ppm, the end point should be reported to within 5 ppm (such as 10-15 ppm). If the compound is effective at a concentration range above 50 ppm, the end point should be reported to within 10 ppm (such as 70-80 ppm).

34. Test cultures of sulfate-reducing bacteria should be used for no more than 1 month. After this period of time, a new test culture must be obtained from the stock culture.

35. Stock cultures should be transferred every 6 months. Stocks can be carried on sulfate-reducing bacteria counting medium (Par. 18) modified to contain 0.3 percent agar.

*A culture which turns black within 24 hours after inoculation.

36. Mid-Continent Strain A† *(Desulfovibrio desulfuricans)* has been designated as the test culture of sulfate-reducing bacteria to be used for comparative screening of chemicals. This culture has been designated NRRL B-4304 and is available from ARS Culture Collection Lab, U.S. Dept. of Agriculture, Northern Regional Research Laboratory, Peoria, Ill. 61604.§

Bacteriostatic Test — Heterotrophic Bacteria

37. Test Organisms:

a. *Pseudomonas fluorescens*‡ (API strain) [NRRL B-4290].§

b. *Bacillus cereus*‡ (API strain) [NRRL B-4278].§

38. The composition of the heterotrophic culture medium for the bacteriostatic test is as follows:

Dextrose (Glucose), grams	0.5
Yeast extract, grams	0.2
NH_4NO_3, grams	3.0
KH_2PO_4, grams	1.0
KCl, grams	0.25
$MgSO_4 \cdot 7H_2O$, grams	0.25
Distilled water, milliliters	1,000.0

Adjust pH to 7.3 with NaOH prior to sterilization.

39. All tests will be performed in adequately covered 18 x 150 mm test tubes. Tubes should be of borosilicate glass. Substitutions of other sizes or material (e.g., plastic) may be made if comparisons with the designated sizes and material show no influence attributable to the alternate choice. Tubes being re-used shall be free of residues which may influence biological or chemical features of the test.

40. The stock cultures of test organisms will be maintained at room temperature on Nutrient Agar or TGE Agar and must be transferred at least once each 30 days.

41. The stock culture will be used to inoculate a tube of sterile test medium 3 or more days prior to test. Each day thereafter another tube of sterile test medium (10.0 ml) will receive 0.1 ml of culture from the 24-hour old tube. The tubes will be incubated at 30 C. The test will be inoculated with a 24-hour old culture representing 3 or more days of successive subculturing.

42. Sufficient inoculum will be used to give a viable count of 5×10^5 organisms per milliliter in the final test solution. The amount of inoculum can be determined by direct microscopic counts with a calibrated

†This culture was isolated by the microbiology laboratory of Texaco Inc.

‡These cultures may be obtained from ARS Culture Collection Lab, U. S. Dept. of Agriculture, Northern Regional Research Laboratory, Peoria, Ill. 61604.

§*NOTE: Users are cautioned not to abuse the permanent storage and culture maintenance courtesy being extended by the U.S. Dept. of Agriculture Northern Regional Research Laboratory by frequently requesting cultures. These are very stable, hardy cultures that can be easily maintained in a viable state using proper media and good microbiological techniques. Alternate sources for culture supply on request have been established as follows: R. L. Huddleston, Continental Oil Co., Research & Development Dept., Ponca City, Okla. 74601; R. L. Raymond, Sun Oil Co., Marcus Hook, Pa. 19061.*

chamber. Other methods for determination of cell densities may be substituted for direct counting if previously calibrated by viable cell counts or by direct counts for each organism grown on the test medium.

43. Stock solutions of the toxicants are to be prepared in sterile distilled water. Each toxicant will be added to a series of clean sterile tubes in sufficient quantity to supply 5, 10, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 ppm to a 10.0-ml volume. However, the toxicant volume shall occupy no more than 0.5 ml. Thereafter, 9.5 ml of inoculated test medium are added. For greatest precision the toxicant volume in each tube should be adjusted to 0.5 ml with sterile distilled water.

44. All tests shall be in duplicate with proper controls.

a. *Chemical control:* Uninoculated tubes should be prepared with one or more of the higher toxicant levels to indicate chemical turbidity of the test medium.

b. *Inoculum control:* The number of organisms per milliliter of toxicant-free test medium should be determined by viable counts with TGE Agar pour plates. Where turbidity due to bacterial growth is questionable, a microscopic examination will be used for confirmation.

45. The results will be reported as the minimum inhibitory range in parts per million for both the 24-hour and 96-hour periods. The report should show the number of organisms at the start of the test and chemical turbidity, if present.

Screening Methods — Other Evaluation Procedures

46. Usually the final evaluation of a compound for use in a particular injection water will involve some variation of the time-kill technique. This type of test involves exposing microorganisms to chemical agents and, at various time intervals, determining the number of surviving organisms. A time-kill test procedure is given in Appendix A.

APPENDIX A

SECTION A-I

APPLICATION OF TIME-KILL PROCEDURE

A1. One application of the time-kill test procedure is as follows:

Injection waters known to be infected with microorganisms are exposed to various concentrations of the chemical under consideration. At specific time intervals, aliquots are removed and placed in suitable counting media (both sulfate-reducer and general counting media). The media are incubated and observed for the number of surviving microorganisms. The test and incubation can be carried out at the temperature of the water in the field. In the time-kill test, the exposure time to the bactericide under consideration should be the same as that used in the field, i. e., the time it takes the water to travel from the point of bactericide injection to the injection formation.

SECTION A-II

SERIAL DILUTION BROTH BOTTLES FOR FIELD CULTURES

Alternative Technique for Estimating Microbial Populations

A2. *Medium:* Standard bacteriological nutrient broth*:

Beef extract, grams	3.0
Peptone, grams	5.0
Distilled water, milliliters	1,000.0

Adjust pH to 7.0 with NaOH prior to sterilization.

A3. *Procedure:* Serum bottles, 10-ml nominal capacity, are filled with 9 ml of the nutrient broth. The bottles are stoppered, using butyl-type rubber stoppers. A disposable metallic cap is used to protect and seal the rubber stopper in place. The filled and sealed bottles are then sterilized at 15 psi steam pressure for 15 min.

A4. Water samples are taken according to the technique described in Par. 4. The first dilution bottle is inoculated with 1 ml of the water sample, using a sterile disposable syringe. The syringe is then discarded. The inoculated bottle is vigorously agitated and, using another sterile syringe, 1 ml of the inoculated broth is withdrawn. This 1 ml of inoculated broth is injected into the second dilution bottle and the procedure is repeated. Subsequent serial dilutions of each water sample are made in the same manner until the dilution factor is at least 10^{-6} (7 bottles). All work should be done in duplicate.

A5. *Incubation:* Incubation may be within 5 C of the water sampled. Bottles which become turbid will be considered positive and probable numbers for the serial dilution bottles will be used to define the general aerobic bacteria population. Dilution bottles are to be held a minimum of 5 days and the data reported as the highest dilution indicating growth, as compared to the lowest dilution showing no growth. The data are reported as a range in numbers, i. e., 10,000-100,000 bacteria per milliliter.

A6. This procedure is recognized as less precise than the plate-count technique. However, it is an [illegible] standard technique (Section I).

*Most biological supply houses.

Alternative Technique for Estimating Sulfate-reducing Bacteria

A7. *Medium:* API sulfate-reducing bacteria medium formula is given in Par. 18.

A8. *Procedure:* Serum bottles are to be 10-ml nominal capacity. Prior to filling and stoppering of the bottles, an iron nail sized to fit the container may be placed in each bottle. If the iron nails are used, the nails should be prepared for use by washing in acetone to degrease them, soaking ½ hour in 2N HCl, washing in water thoroughly to remove all acid, and then transferring into a container of acetone for storage. If preferred, the nails may be sand-blasted prior to use instead of using acid etch. Iron wire or reduced iron powder, reagent grade, may be substituted for the iron nail.

A9. In order to exclude oxygen contamination, the bottles can be filled with 9 ml of hot broth, stoppered and capped or blanketed under an inert gas atmosphere. The stoppers are to be of butyl-type rubber, and the caps are to be disposable metallic covers. The bottles and contents are sterilized at 15 psi steam pressure for 15 min, after the metal caps have been sealed.

A10. Disposable, one-time use, pre-sterilized syringes are to be used. Water samples are to be taken according to the technique described in Par. 4. The first dilution bottle will be inoculated with 1 ml of the water sample. Subsequent dilutions may be made by vigorously agitating the sample; then, using a sterile syringe, withdraw 1 ml of the inoculated broth. This 1 ml of inoculated broth is injected into the next dilution bottle. The syringe is then discarded and the procedure is repeated. Serial dilutions are run to 10^{-4}.

A11. *Incubation:* Incubation may be within 5 C of the water being sampled. Bottles which turn black will be used to define the sulfate-reducing bacteria population. Bottles which turn black within 2 hours are not to be considered positive since this probably will be due to the presence of sulfide ion in the sample. Subcultures of these false positive samples may be made after 1 week. Cultures are to be held a minimum of 28 days, and the data reported as the highest dilution indicating growth, as compared to the lowest dilution showing no growth. The data are reported as a range in numbers, i.e., 100-1,000 sulfate-reducing bacteria per milliliter.

A12. This procedure is recognized as an acceptable alternative technique to the standard technique (Section I) for culturing sulfate-reducing bacteria when the technique is used in the field. It is recognized that the method has limitations and that results may vary from data obtained by Section I standard techniques.

APPENDIX B

SPECIAL APPARATUS

Bacteria-colony counter darkfield

Bacteria-counting chamber—Petroff-Hausser type

Cover glasses—round or square

Crimping tool—for serum-bottle aluminum closures

Incubator—constant temperature

Microscope—capable of providing magnification from 100 to 1,000 diameters (preference, phase contrast)

Petri dishes—100-mm diameter

Pipettes—bacteriological or seriological 1- and 10-ml

Slides—standard and hanging drop

Sterilizer—steam pressure

Test tubes—bacteriological (without flare), 150 x 18 mm, or with screw caps with foil, rubber, or teflon liners

Water bath—constant temperature

Membrane filters and holders

Sample bottles—6- to 8-oz.

Bottles—1- or 2-oz clear

Bottle screw caps—liners of aluminum foil or teflon

Filter paper—Whatman No. 1 or No. 2, or equivalent

Serum bottles—10-ml nominal capacity with butyl rubber stoppers and aluminum closures

Syringes—sterile disposable with needle