Rapid Field Method for the Detection and Enumeration of Sulfate Reducers

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FROM: Barry, Sheila and D.R. Houghton (Eds.), 1986. Biodeterioration 6, Proceedings of the Sixth International Biodeterioration Symposium. C.A.B. Intl. Mycological Inst., The Biodetn. Soc., United Kingdom.

Summary

Laboratory experiments were conducted to evaluate a relatively new rapid method for the enumeration of sulfate-reducing bacteria. The method uses an agar deep containing a modified version of Sulfate API Agar and a unique sampling applicator. The method was compared with Sulfate API Agar using a two-inch length of capillary tubing as a sampling applicator. The production of iron sulfide precipitate was used as an indicator of sulfate reducer growth.

Metalworking emulsions contaminated with sulfate reducers as well as a broth culture of *Desulfovibrio desulfuricans* (Mid-Continent Strain A) were tested. Under all conditions, the modified medium provided faster results. Because the test applicator holds a sample volume almost ten-fold greater than the capillary (0.3 ml vs. 0.04 ml) and because of ease of handling, it was judged to be superior to the capillary. Use of the modified medium with a unique applicator yielded positive results often several days earlier than Sulfate API Agar with a capillary tube sampler.

I. Intoduction

Sulfate-reducing bacteria such as *Desulfovibrio desulfuricans* are ubiquitous in nature and are often involved in the biodeterioration of materials. Frequently they have caused problems in oilfield injection waters, cooling waters and metalworking fluids (Rossmoore *et al.* 1964, Hill 1975). Unchecked growth of these organisms can result in corrosion of metals, pitting of concrete and foul odors.

Commonly used methods for the detection and enumeration of sulfate-reducing bacteria are often time consuming and cumbersome and thus are not suited for field use. *Standard Methods for the Examination of Water and Wastewater* (1981) describes a two-step method involving membrane filtration which takes from 4 to 21 days for a positive reaction. The American Petroleum Institute in their *Recommended Practice for Biological Analysis of Subsurface Injection Waters* (American Petroleum Institute 1975) outlines a technique for estimating sulfate-reducing bacteria in the field. This method uses a syringe to make multiple dilutions into serum bottles containing iron nails, and cultures must be held a minimum of 28 days for complete reporting.

A procedure for field use should be easy to handle and give a relatively fast reaction for those samples containing sulfate-reducing bacteria. To aid in ease of handling, we used a unique applicator that could deliver a large volume of sample into a relatively small amount of medium. Multiple dilutions are not necessary since the rate of iron sulfide production is proportional to the number of sulfate reducers introduced into the medium. Comparisons of sulfate reducer culture media have shown that various modifications of commercial formulae could be used to shorten reaction times (Mara & Williams 1970). By supplementing a known medium, Sulfate API, more rapid positive reactions were achieved compared to the standard formula.

II. Materials and Methods

Culture Media

We compared two culture media formulations. One was Bacto Sulfate API Agar (DIFCO Laboratories, Detroit, MI) (API) and the other was a modification of API medium referred to as SR Medium (SR). The per liter formula is as follows:

	SR	API
Yeast extract	1.0 g	1.0 g
Ascorbic acid	0.1 g	0.1 g
Sodium lactate	5.2 g	5.2 g
Sodium sulfite	0.6 g	—
Magnesium sulfate	0.2 g	0.2 g
Dipotassium phosphate	0.01 g	0.01 g
Ferrous ammonium sulfate	0.2 g	0.1 g
Sodium chloride	10.0 g	10.0 g
Agar	7.0 g	15.0 g

The differences between the SR medium and the API medium are that the SR medium contains 0.6 g sodium sulfite while the API has none, and SR contains double the ferrous ammonium sulfate and about half the agar of the API formula. Each medium was dispensed into eight-milliliter glass vials.

Samples Evaluated for Sulfate-Reducing Bacteria

In order to compare the rates of reaction in the various test systems and their relative sensitivities, three samples were tested undiluted and diluted 1:10 and 1:100. Two of the samples were metalworking soluble oils, one of which contained 4 x 10^6 sulfate reducers per ml and the other 10^5 sulfate reducers per ml. The third sample was a broth culture of *D. desulfuricans* (Mid-Continent Strain A) that contained 6 x 10^7 CFU/ml. The sulfate reducer population of each sample was established using a shake tube technique (Mara & Williams 1970).

Comparison of Test Methods

Volumes of 0.3 ml and 0.04 ml were sampled from each dilution of the soluble oils and the broth culture. Each volume was obtained by two different methods. The 0.3 ml volume was taken in the following ways. Pipe cleaners were cut in two-inch lengths to fit the 8 ml agar deeps. The two-inch piece of pipe cleaner absorbs approximately 0.3 ml of liquid sample. The pipe cleaners were held with forceps, immersed into the sample, and then stabbed into the agar. The 0.3 ml aliquot was also sampled without the pipe cleaner. A plug of agar having the approximate diameter of the pipe cleaner was removed from the deep, and 0.3 ml of sample was injected into this space using a Pasteur pipet. In both cases several drops of mineral oil were added to each tube. The tubes were then tightly capped and incubated at 30°C and observed at regular time intervals for the production of iron sulfide precipitate.

The 0.04 ml volumes were sampled in the following ways. Two-inch lengths of capillary tubing containing approximately 0.04 ml were held by forceps and immersed into the sample. The liquid was allowed to fill the capillary and the filled tube was stabbed into the agar deep. The second method used to sample 0.04 ml involved spreading 0.04 ml of the liquid onto a two-inch pipe cleaner which was then stabbed into the agar. Again, several drops of mineral oil were added to each deep before they were capped and incubated.

The amount of sulfate reducer growth was measured using the degree of blackening of the agar deep as an indicator. Fig. 1 shows the agar deeps with the designation given to each degree of reaction. The designations were arbitrarily established so that accurate comparisons could be made between degrees of reaction. We examined the tubes at 18, 24, 40, 48, 60, 72, 90, 138 and 168 hours (one week) for the degree of reaction. There was a total of eight conditions:

API agar+pipe cleaner+0.3 ml sample
API agar+pipe cleaner+0.04 ml sample
API agar+capillary+0.04 ml sample
API agar+0.3 ml sample (via Pasteur pipet)
SR agar+pipe cleaner+0.3 ml sample
SR agar+pipe cleaner+0.04 ml sample
SR agar+capillary+0.04 ml sample
SR agar+0.3 ml sample (via Pasteur pipet)

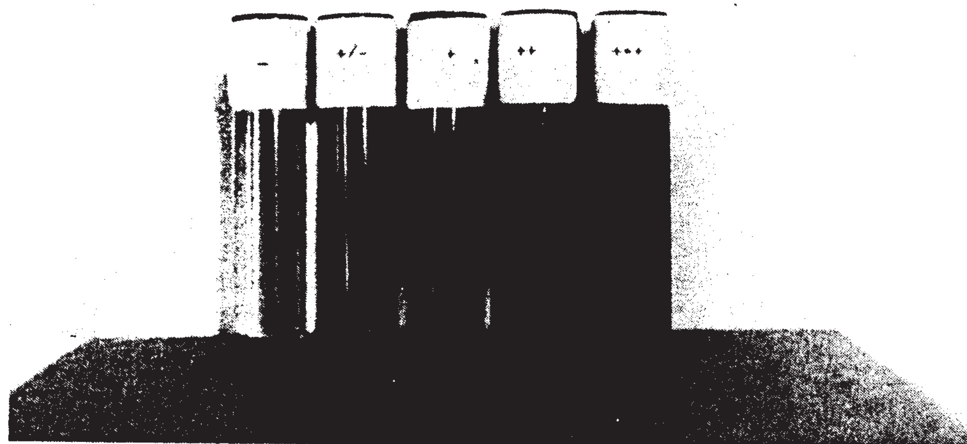


Fig. 1. Semisolid agar deeps showing varying degrees of blackening resulting from iron sulfide precipitation. KEY - no growth. +/ - very slight growth/greying around inoculation site. + light growth/blackening around site or bottom of tube. ++ moderate growth/entire tube light grey or heavy blackening at inoculation site. +++ entire tube black.

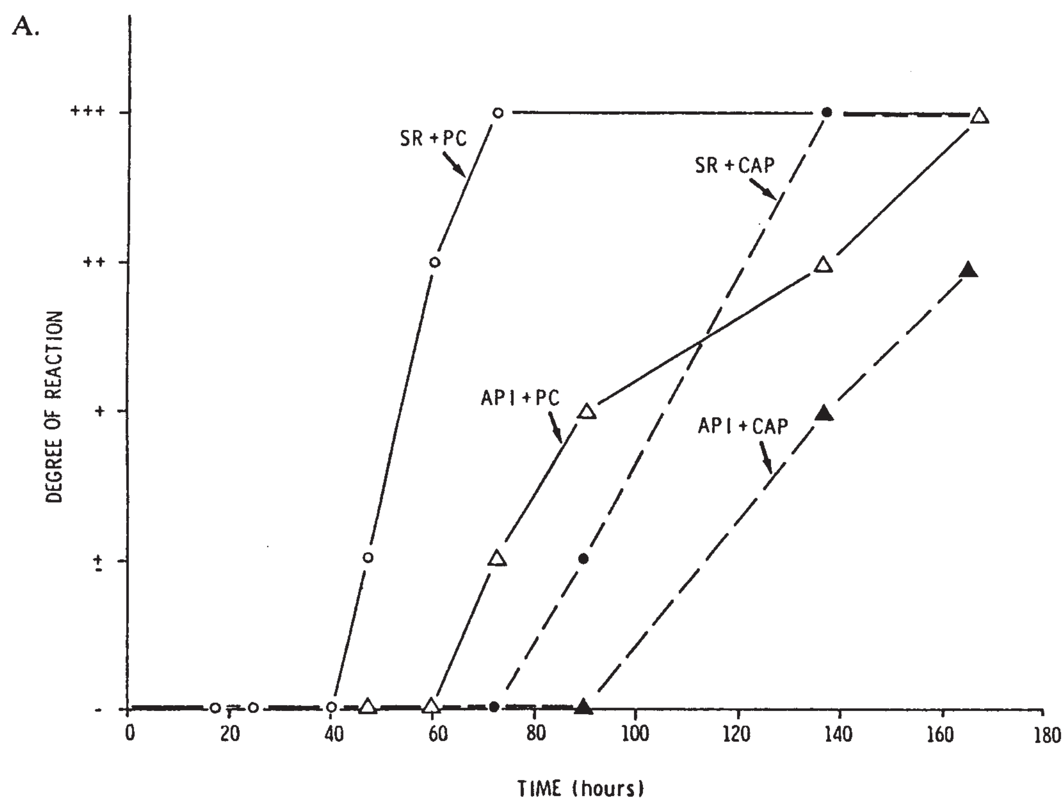
III. Results and Discussion

The results showed that the samples, regardless of dilution, followed the same trends. Therefore, to avoid redundancy, we will report only the results of the 1:10 dilution. Fig. 2A, 2B and 2C show results from one of the metalworking emulsions. Fig. 2A shows a comparison of SR medium when used with a pipe cleaner (PC) (0.3 ml) and a capillary tube (CAP) (0.04 ml) and API medium under the same conditions. At 48 hours, the only condition to generate a positive reaction was the SR + PC. At 72 hours, the API + PC was slightly positive. At 90 hours, the SR + CAP was positive, and at 132 hours, the API + CAP reacted.

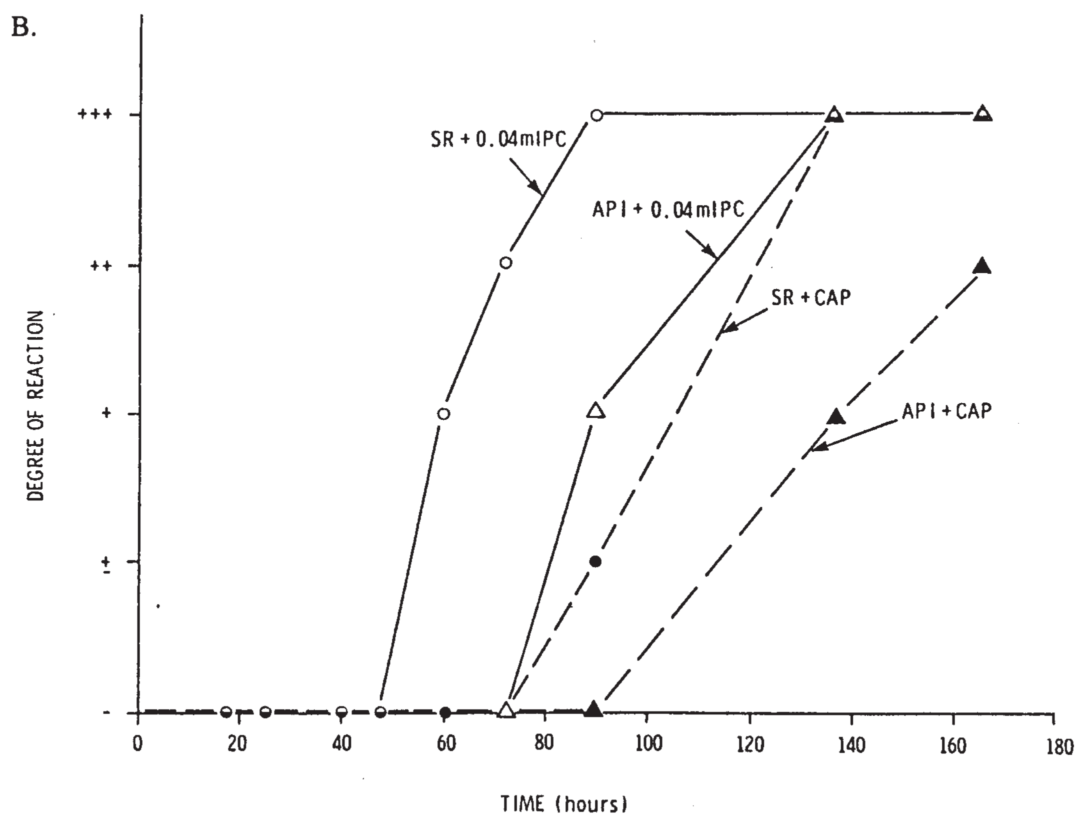
Since the pipe cleaner holds almost ten times the sample volume as the capillary, it is not surprising that the capillary reacted more slowly. Fig. 2B shows that even when equal volumes are compared, the pipe cleaner is still superior to the capillary in terms of quickness of reaction.

Fig. 2C is a further effort to evaluate the role of the pipe cleaner beyond its transfer of a larger sample volume. As in 2B, equal volumes are compared except a sample volume of 0.3 ml instead of 0.04 ml is used, and pipe cleaner vs. no sample apparatus are compared rather than pipe cleaner vs. capillary. Fig. 2C shows that there is a slight difference in reaction times between SR + PC and SR + 0.3 ml and a more considerable difference between API + 0.3 ml and API + PC. In both cases the injection of sample into

A.



B.



C.

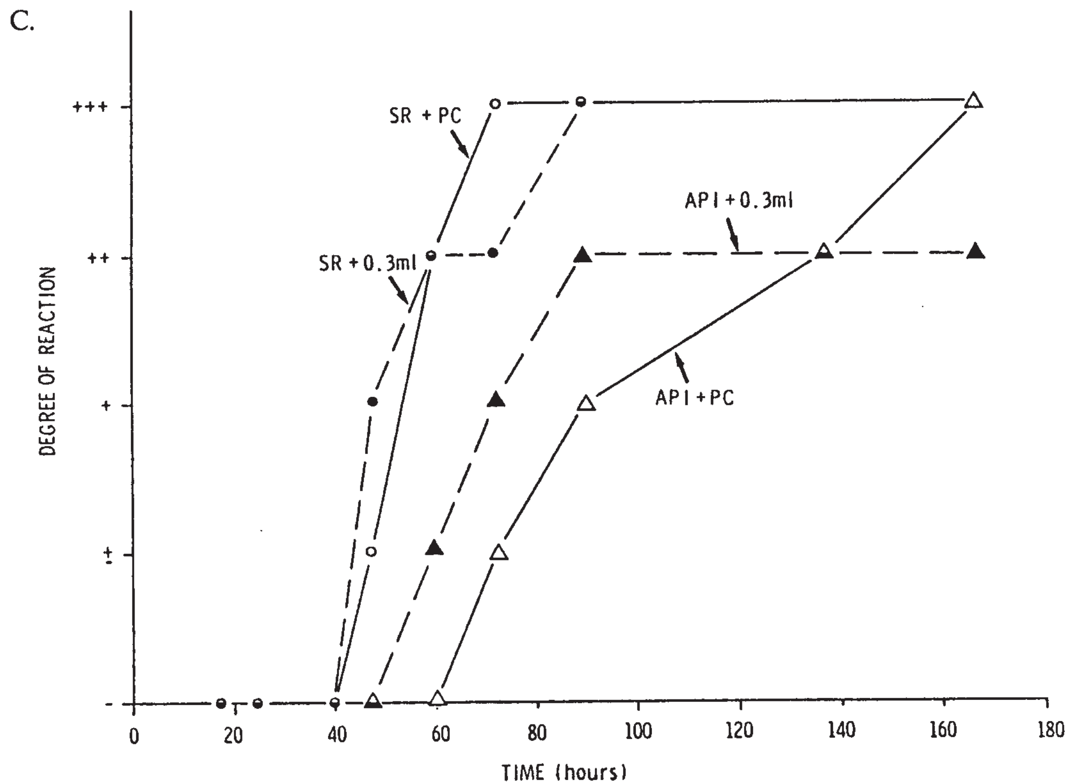


Fig. 2. Comparison of media and methods for detecting sulfate reducers in a contaminated soluble oil. The samples were a 1:10 dilution of a soluble oil containing 10^5 sulfate reducers per ml. *A:* Comparison of modified medium (SR) vs. API medium (API) and pipe cleaner applicator containing 0.3 ml (PC) vs. a capillary containing 0.04 ml (CAP). *B*: Comparison using a constant 0.04 ml sample volume. *C:* Comparison using a constant 0.3 ml sample volume.

the medium via a Pasteur pipet gave a slightly quicker reaction time than the use of the pipe cleaner. This comparison is valid, but for field use it would be difficult to remove an agar plug in order to be able to inject a 0.3 ml sample volume. Therefore, considering the ease of using the pipe cleaner as a sampler compared to the Pasteur pipet, the difference in reaction times is negligible. In addition, the pipe cleaner can be used for sampling solid surfaces and viscous liquids that would not be suitable for pipetting.

Fig. 3A shows the results of using a 1:10 dilution of a broth culture of *D. desulfuricans*. The trends here are the same as with the soluble oil sample using the same conditions (Fig. 2A). The SR + PC combination was positive in 18 hours, the API + PC took 40 hours, the SR + CAP was positive in 48 hours, and it took one week for the API + CAP to show a positive reaction.

Fig. 3B is a comparison of the two media when a constant 0.3 ml volume is used. We included this condition to determine if the pipe cleaner, either by configuration or content, stimulated or inhibited growth. The results show it is the relatively large sample volume the pipe cleaner is able to deliver which is its major advantage. There is virtually no difference in result when 0.3 ml is inoculated via pipe cleaner or pipet. As mentioned, there is a difference in ease of test performance between the pipe cleaner and the Pasteur pipet.

Overall, we found the use of the SR formula and the pipe cleaner (with 0.3 ml) to be far superior to the other seven test conditions. It was the easiest to use and consistently provided rapid results. The pipe cleaner allows for the pickup and delivery of a relatively large sample size in a one-step procedure. The modification made in Bacto Sulfate API Agar (DIFCO Laboratories, USA) greatly increased the sensitivity of the medium and allowed for reaction times to be substantially shortened.

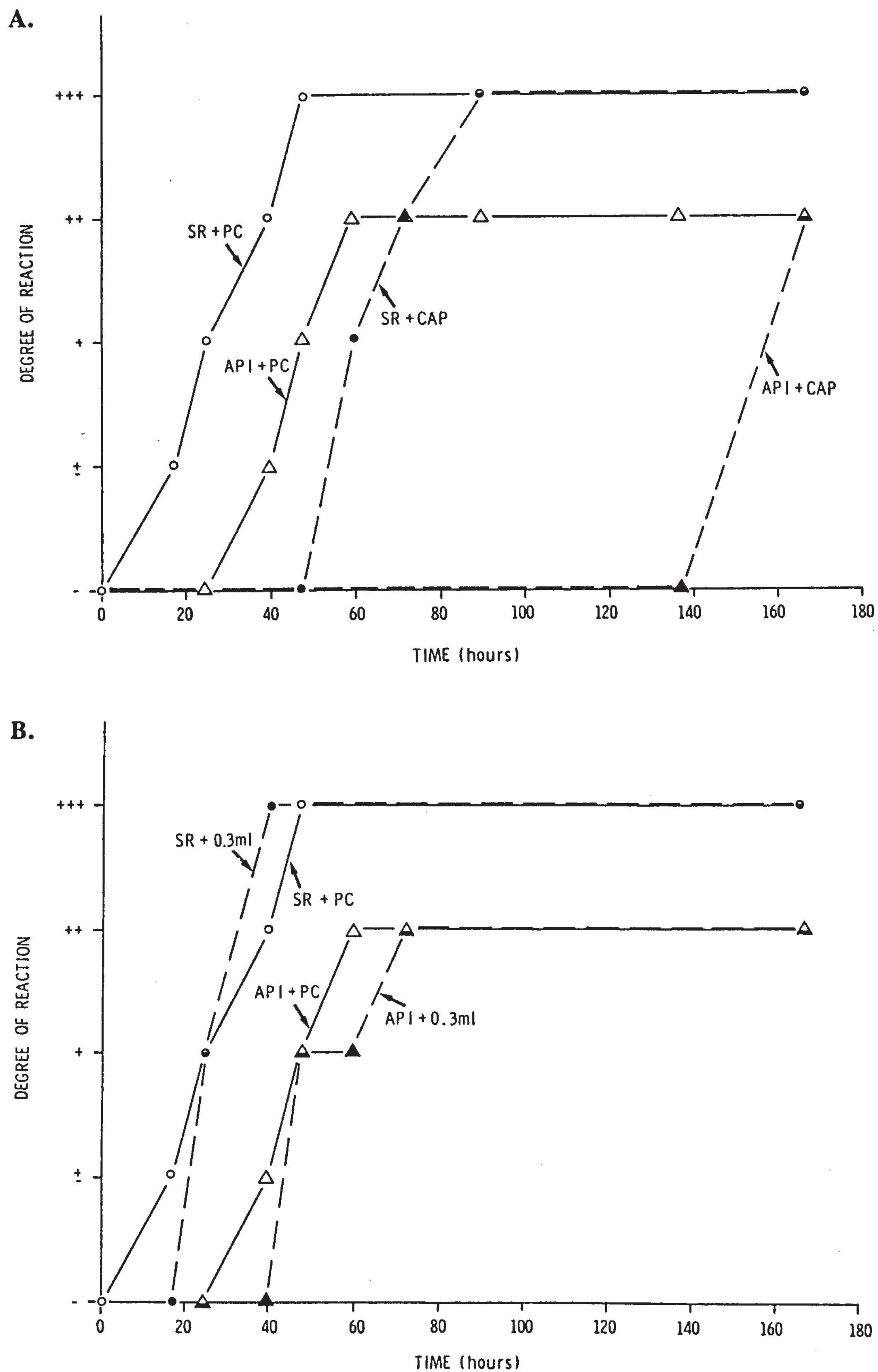


Fig. 3. Comparison of media and methods for detecting sulfate reducers in a broth culture of *Desulfovibrio desulfuricans*. The samples were a 1:10 dilution of a culture containing 6×10^7 sulfate reducers per ml. *A*: Modified medium vs. API medium and PC (0.3 ml) vs. CAP (0.04 ml). *B*: Comparison using constant 0.3 ml sample volume.

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