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ALTERNATIVE METHOD FOR MONITORING THE EFFECT OF INHIBITORS ON SULFATE REDUCTION

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A radiotracer technique using $^{35}SO_4$ is presented as an alternative method for testing the efficacity of inhibitors of sulfate-reducing bacteria under environmental conditions. This method is compared with two other conventional techniques for evaluating biocide efficacity by testing micro-organism survival. Differences between the methods are described. The radiotracer technique gives a direct measure of the sulfate reduction rate and by using it the effect of inhibitors can be evaluated in situ. This method should be considered when choosing a test for the effects of biocides.

The negative influence of bacterial sulfate reduction on mankind's economy is well documented and occurs in a large number of ways (*1–4*). Pollution, corrosion of metals and stonework, food spoilage and problems in oil technology are only a few examples of its many forms. Another problem caused by sulfate-reducing bacteria occurs when petroleum products stored in steel tanks become contaminated by sulfur compounds due to sulfide-production (*5*). We have observed such effects in rock caverns where jet fuel has been stored on a waterbed (*6*). These problems have of course raised the question of how it would be possible to inhibit the sulfide production. Considerable research has been devoted to testing various potential microbial biocides against sulfate-reducing bacteria (*5*, *7–12*). Most of these studies have used techniques for counting the numbers of viable bacteria when evaluating the effects of inhibitors. For such studies these techniques has several major limitations:

1) There are no universal media for growing all types of sulfate-reducing bacteria.

2) Sulfate-reducing bacteria have a tendency to form aggregates causing underestimation of the actual number of bacteria.

3) There is always the risk that bacteria grown in enrichment cultures may not be active in the natural environment.

4) A direct correlation does not necessarily exist between the production of hydrogen sulfide and the number of bacteria.

HARDY and SYRETT (*9*) have recently published a radiorespirometric method for evaluating inhibitors of sulfate-reducing bacteria. In their study they use $^{35}SO_4$ to follow the inhibition of the sulfide production in synthetic growth media. It is also possible to follow the reduction rate of sulfate directly in an environmental sample by using a radiorespirometric method (*13*, *14*).

A method by which the sulfate reduction rate can be followed after addition of inhibitors to a specific environmental sample, can be used to determine the effectiveness of the compounds in inhibiting the sulfate-reduction in situ. This approach to testing biocides can be advantageous in many situations, especially in environments where the microorganisms are difficult to enumerate accurately.

The aim of the present study was to demonstrate the use of such a radiotracer technique when investigating some inhibitors' effects on the sulfate reduction rate in sediment from a rock cavern used for oil storage. Also the results obtained using the radiotracer technique were compared with other methods using bacterial counts.

MATERIALS AND METHODS

Characterisation of sediment samples. The sediment samples were collected from a recently emptied rock cavern in southern Sweden, previously used for storing jet aviation fuel. The sediment had a water content of 29 %, E_h of −106 mV, pH 6.2 and the amount of organic matter was 1.3 %. The in situ sediment temperature varied from 4 to 8° during the year. The sulfate concentration was 20 μg per gram of sediment (dry weight) and the sulfide concentration was 1 mg per gram of sediment (dry weight). During the inhibition experiments extra sulfate was added to a final concentration of 100 μg sulfate per gram of sediment (dry weight).

Quantification of sulfate reduction rate in sediments. Sediment (one gram, dry weight) homogenized in 1.1 ml water from the rock cavern was dispensed anaerobically into a 10-ml serum vial. The headspace was then flushed with oxygen-free N_2 after which the vial was stoppered with a rubber stopper and sealed by crimping with an aluminium seal. Sodium (^{35}S) sulfate solution (100 μl, 20 μCi/ml, 66 mCi/mmol) was injected through the stopper with a 1-ml syringe. The sediment-isotope mixture was blended vigorously and incubated in the dark at 4°. Incubation was stopped by adding 1 ml 1.4 M zinc acetate to each vial and then freezing it instantly at −20° pending analysis. After thawing, the incubated sample was connected to another 10-ml rubber-stoppered serum vial containing 4 ml 1.4 M zinc acetate. This latter vial had been flushed with oxygen-free N_2 and its gas oultet was a syringe mounted in the rubber stopper and filled with 1 ml 1.4 M zinc acetate (Fig. 1). The sediment was then acidified with 6 N

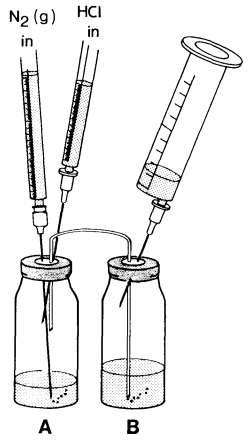

Fig. 1. Quantification of sulfate reduction in sediments. Bottle A is the vessel in which the sample is incubated. Bottle B is filled with 4 ml 1.4 M zinc acetate and a syringe filled with 1 ml 1.4 M zinc acetate is mounted on the top of the bottle for gas outflow.

HCl and the liberated H_2S gas was quantitatively recovered in the zinc acetate by flushing with oxygen-free N_2. After flushing, the absorption solution in the syringe was added to the absorption vessel. The precipitated zinc sulfide was oxidized to zinc sulfate according to the method of TAYLOR et al. (*15*). A 1-ml sample was then mixed with 10 ml of Ready-solv EP scintillation cocktail (Beckman) to determine the amount of produced radioactive sulfide. The slurry remaining in the reaction vessel was diluted with 3-ml of double-distilled water and centrifuged. A 1-ml sample of the supernatant was used to quantify the remaining radioactive sulfate. The sulfate reduction rates were calculated as described by JØRGENSEN (*16*).

Quantification of the degree of inhibition. Multiple homogenized sediment samples were incubated as described above but before sealing the serum vials, the inhibitor to be tested was added at different concentrations. Four serum vials were used in parallel for each experimental treatment. In order to follow the time dependence of the inhibitor's efficacity, $^{35}SO_4$ was added to the different reaction vessels at one, four and seven days after the addition of the inhibitors. Incubation and analysis of the samples were performed as described above. Autoclaved control samples were also incubated and analysed in parallel with the inhibitor samples.

Sulfide precipitation by added inhibitor. Before testing the inhibitor's efficacity on sulfate reduction in the described test system a check was made to see whether sulfide was precipitated by the inhibitor or not. Sediment samples were mixed with the different concentrations of inhibitors that were to be used in the inhibition study. The amount of H_2S gas liberated after acidification was de-

termined chemically, using the methylene blue method according to FOGO and POPOWSKY (*17*), and compared with the amount of H_2S gas liberated from a sample where no inhibitor was added. When sodium molybdate was tested 100 mM of a titanium chloride solution was added immediately before acidification to avoid reoxidation of the sulfide in the presence of molybdate (*13*).

Chemical analyses of sediments. The sulfide measurements were made essentially according to the method of FOGO and POPOWSKY (*17*). The method of TABATABAI (*18*) was used for sulfate determination.

The dry weight of the sediment was determined by weighing samples of sediment before and after heating at 105° for 24 hr.

The organic matter content was determined by measuring the loss of weight on ignition at 475° for ca. 5 hr in a muffle furnace (*19*).

Estimation of bacterial counts in sediments. One gram of sediment (dry weight) was dispensed in 5 ml of sterile 0.9% NaCl and succesively diluted. The numbers of sulfate-reducing bacteria in the sediments were determined by the most probable number (MPN) method with five tubes per dilution using POSTGATE's medium B (*20*).

Test of the effect of inhibitors in synthetic growth media. This test was performed essentially according to SALEH et al. (*12*). Serial dilutions of filter sterilized stock solutions of the inhibitors were made in sterile water and 1 ml of the appropriate inhibitor solution was added to 9 ml of medium B (*20*). For controls distilled water was added in place of the inhibitor solution. A diluted sediment sample of 1 ml in which the number of sulfate-reducing bacteria was about 2×10^3 bacteria/ml, was added to each tube. Triplicate tubes were employed for each experimental treatment. The tubes were incubated at 30° and were examined after 14 days of media blackening (formation of FeS).

Chemicals. $Na_2{}^{35}SO_4$ (66 mCi/mmol) was purchased from the Amersham Radiochemical Centre. The isothiazolone compound (5-chloro-2-methyl-4-isothiazolin-3-one, 2-methyl-4-isothiazolin-3-one) was obtained from Rohm and Haas Nordiska AB, Bromma, Sweden. All other chemicals used were of reagent grade.

RESULTS

The effect of different inhibitors on sulfate reduction

Sulfate reduction was studied in homogenized sediment samples which were incubated with inhibitors at different concentrations. The effects of the inhibitors on the numbers of sulfate-reducing bacteria were followed during a period of one week where sediment samples were tested for bacterial counts using the most probable number method (MPN) at day one, day four and day seven.

The inhibitors used were cupric nitrate, an isothiazolone compound, and two sulfate structure analogues, sodium molybdate and sodium selenate. Sodium

Table 1. The effect of inhibitors on the number of sulfate-reducing bacteria in sediment.

Inhibitor	Inhibitor concentration (μg/g sediment (dry weight))	Time (days) 1	4	7
		SRB[a]	SRB[a]	SRB[a]
Sodium selenate	0	2×10^4	2×10^4	2×10^4
	1	6×10^3	1×10^4	2×10^3
	10	2×10^4	3×10^4	1×10^4
	100	6×10^3	2×10^4	1×10^5
	1000	2×10^3	4×10^3	1×10^5
Isothiazolone compound	0	2×10^3	2×10^4	3×10^4
	10	2×10^3	8×10^3	2×10^4
	50	2×10^3	2×10^4	2×10^4
	100	2×10^3	7×10^3	9×10^3
Sodium molybdate	0	2×10^4	2×10^3	3×10^4
	10	1×10^4	4×10^3	1×10^4
	100	4×10^4	2×10^3	4×10^4
	1000	6×10^4	2×10^3	8×10^3
Cupric nitrate	0	3×10^4	4×10^5	4×10^5
	1000	2×10^4	1×10^5	1×10^4
	10000	2×10^4	4×10^4	2×10^4
	50000	1×10^4	4×10^4	6×10^4

[a] Sulfate-reducing bacteria/gram sediment (dry weight).

molybdate (*13*) and sodium selenate (*21*) have been shown to inhibit sulfate reduction in environmental sediment samples. Cupric nitrate was included in the experiment to study the combined inhibitory effect of cupric ions and nitrate ions on the sulfate reduction. The isothiazolone compound used has been shown to inhibit sulfate reduction (*22*) and its biodegradability (*23*) makes it an interesting biocide to use on a larger scale.

Table 1 shows the results of these inhibitors using the bacterial count method. As can be seen, this method showed no significant effects of the inhibitors. It is notable, however, that the inhibitors used in this study are bacteriostatic compounds, so the effect of the biocides are probably lowered due to dilution when samples are prepared for bacterial counts.

To overcome this problem another test using bacterial counting was performed in which the inhibitors at different concentrations were added directly to a synthetic growth medium for sulfate-reducing bacteria. After inoculation of a diluted sediment sample the effect of the inhibitors was analysed. The results (Table 2) gives a rough estimation of the effects of the inhibitors in a synthetic growth medium. However, it is very difficult to relate the effects in a synthetic growth medium to the in situ situation.

Therefore the third method was tested based on measuring the sulfate reduction activity in the sediments using the radiotracer technique. These experi-

Table 2. The effect of inhibitors on the growth of sulfate-reducing bacteria in synthetic growth media (POSTGATE's medium B).

Sodium selenate		Sodium molybdate		Cupric nitrate		Isothiazolone compound	
Concentration (μg/tube)	Growth	Concentration (μg/tube)	Growth	Concentration (mg/tube)	Growth	Concentration (μg/tube)	Growth
0	+	0	+	0	+	0	+
1	+	10	+	1	+	10	−
10	+	100	−	10	−	50	−
100	−	1,000	−	50	−	100	−
1,000	−						

+: blackening, −: no blackening.

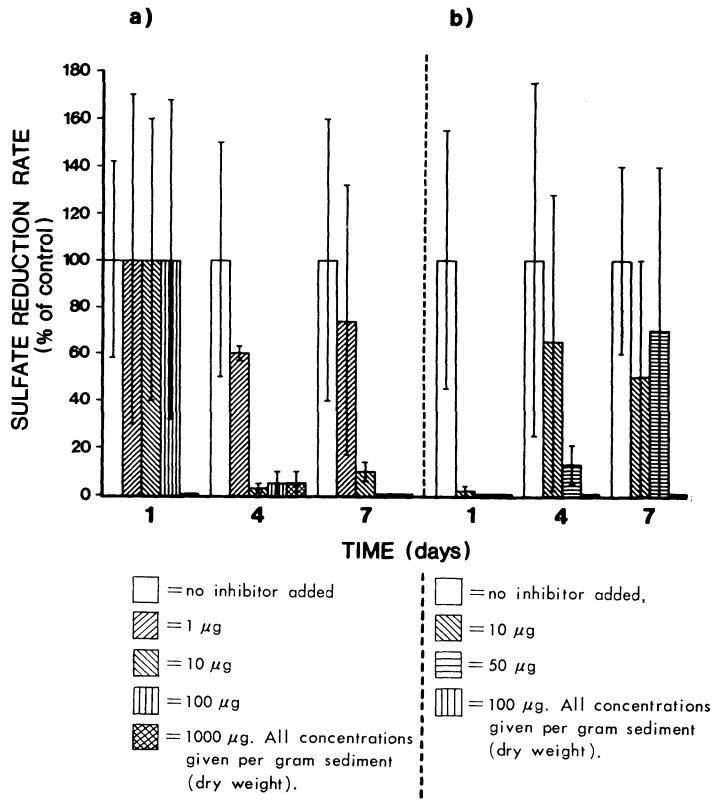

Fig. 2a. Effect of sodium selenate on the sulfate reduction rate.

Fig. 2b. Effect of a isothiazolone compound on the sulfate reduction rate.

ments were performed exactly as the sediment experiment described above except the sulfate reduction activities were measured instead of bacterial counts.

The results (Fig. 2a–d) are expressed as percentage of sulfate reduction rates

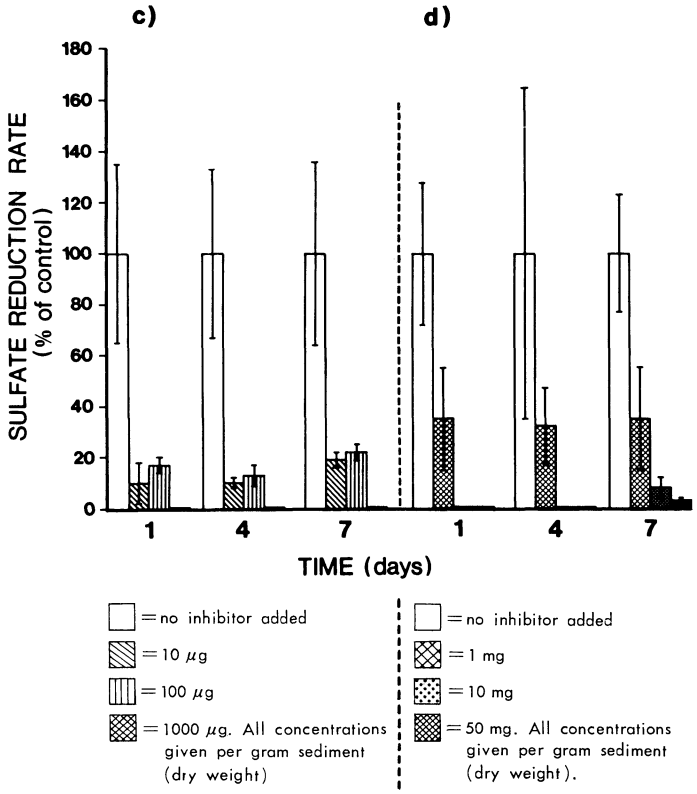

Fig. 2c. Effect of sodium molybdate on the sulfate reduction rate.

Fig. 2d. Effect of cupric nitrate on the sulfate reduction rate.

in samples with added inhibitor compared with samples with no inhibitor. The mean value of the sulfate reduction rate in samples where no inhibitor was added was 1×10^{-8} mol of sulfate per day and gram of sediment (dry weight). The variability in the measurements are expressed as the standard deviation calculated from the reduction rate of the different samples. As can be seen from the results, the time-dependent effect on the reduction rate of the two sulfate structure analogues, molybdate and selenate, are quite different. Molybdate seems to inhibit the reduction rate immediately (within 24 hr) while selenate inhibits it gradually. There are some concentrations, i.e. 10 or 100 μg of sodium molybdate that give a reduced sulfate reduction rate. However, the inhibitory capacity of these two sulfate analogues were rather constant during the experiment. Another result concerning the long-term effects of biocides on sulfate reduction is achieved in the tests with cupric nitrate and the isothiazolone compound. Both these compounds seem to have only a temporary inhibitory effect at low concentrations while higher amounts give a prolonged effect, though not

as good as for the sulfate analogues tested.

In the radiotracer studies, precipitation tests were made in order to ensure that no artificial results were achieved because of specific precipitation of sulfide by the inhibitors. None of the inhibitors precipitated sulfide. However, with molybdate it was necessary to add titanium chloride to the reaction vessels before acidification in order to avoid reoxidation of sulfide.

DISCUSSION

The problems caused by sulfate-reducing bacteria have been known for a long time. Many attempts have been made to inhibit these bacteria and their disturbance of mankind's economical activities. The classical way of testing inhibition has been to count bacterial numbers. This has been done either by adding the inhibitor to an inoculated synthetic medium or by adding the biocide to an ecological sample and then count the bacteria in the sample by conventional techniques. In using these methods, the results depend to a great extent on which medium and inhibitor is being used. If the aim is an all-or-none response of growth after the treatment with a bactericidal inhibitor, the interpretation of the results is still difficult because of the uncertainty as to the number of bacteria growing in the medium used in the test. For instance, to our knowledge all the inhibition studies performed so far have concentrated on the effect of biocides on lactate-utilizing sulfate-reducing bacteria while studies on fatty-acid utilizers have been neglected.

A new approach to testing inhibitors of sulfate-reduction was recently published by HARDY and SYRETT (*9*). They used a radiotracer technique with which they followed the sulfide production in the presence of different inhibitors. They showed that the method was both rapid and sensitive and also relatively simple. However, they performed their inhibition studies by inoculating the bacteria into a synthetic growth medium. But the problem remains when the testing is done in a synthetic medium, how the results can be used to predict the effect of the inhibitor in situ.

Using the radiotracer technique directly in the environmental sample probably gives more pertinent information since it measures the actual systems of interest and measures them in situ. However, there are some questions that have to be considered when using this method. Firstly the sulfate concentration in the sample is important. It is neccessary to correlate the incubation time in the test to the sulfate concentration. If the concentration is low, the incubation time should be short. This is to ensure that the measured sulfate reduction rate follows the kinetics of the first order. If the incubation time is too long the measured value will be an underestimation of the real value because of sulfate limitation. For that reason, in this study, we added extra sulfate to our sediment, since the sulfate concentration in the sediments was very low. Another

question to be considered is the system being studied. We have been using rock cavern sediment because we are interested in sulfate-reducing bacteria in that situation. However, sediment samples are difficult to handle and, as can be seen in Fig. 2, the results may vary somewhat between different samples at the same inhibition concentration. This may be due to the difficulties in getting homogeneous multiple samples, and the uncertainty of such small systems behaviour during longer incubation times. However, this method gives significant indications of how well the biocides are working in situ. If systems with a higher water content are studied the results achieved probably would not vary so much.

Comparing the radiotracer technique with the bacterial count technique is of course difficult especially as in this study only bacteriostatic inhibitors have been used. Still these results point to another problem arising in inhibition studies using bacterial counts.

The test where inhibitors were added directly to a growth medium for sulfate-reducing bacteria gave the same conflicting data as the activity measurements. These experiments give only an all-or-none response on the efficacity of the inhibitors. Conclusions drawn from such results may therefore be wrong for instance due to the use of the wrong media (different types of sulfate-reducing bacteria are not susceptible to the same inhibitors).

The main conclusion of this paper is that the use of the $^{35}SO_4$ radiotracer technique alone or as a complement should be seriously considered for use in studies on the effect of biocides on sulfate-reducing bacteria.

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