A Rapid and Simple Method for Estimating Sulfate Reduction Activity and Quantifying Inorganic Sulfides

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A simplified passive extraction procedure for quantifying reduced inorganic sulfur compounds from sediments and water is presented. This method may also be used for the estimation of sulfate reduction rates. Efficient extraction of FeS, FeS₂, and S²⁻ was obtained with this procedure; however, the efficiency for S⁰ depended on the form that was tested. Passive extraction can be used with samples containing up to 20 mg of reduced sulfur. We demonstrated the utility of this technique in a determination of both sulfate reduction rates and reduced inorganic sulfur pools in marine and freshwater sediments. A side-by-side comparison of the passive extraction method with the established single-step distillation technique yielded comparable results with a fraction of the effort.

Sulfate-reducing bacteria are nearly ubiquitously distributed and are major contributors to the degradation of organic matter in both freshwater and marine environments (11). These organisms are known to catalyze desirable processes such as the biodegradation of pollutant chemicals (10). They are also involved in undesirable processes including the souring of oil formations and the corrosion of steel equipment (2). Techniques for the examination of sulfate reduction have led to better understanding, application, and monitoring of this important process.

As a group, sulfate-reducing bacteria are extremely versatile with the ability to metabolize diverse organic and inorganic electron donors (3, 18). They are also known to utilize a wide variety of electron acceptors (nitrate, iron, oxygen, uranium, thiosulfate, elemental sulfur, and halo- or nitro-aromatic compounds) either in the absence or sometimes in the presence of sulfate (9, 17, 20). Given their metabolic diversity, the mere presence of the requisite bacteria does not necessarily coincide with sulfate-reducing activity. Furthermore, while the consumption of sulfate and the production of sulfide are indicative of sulfate reduction, the absence of changes in these chemical species cannot be taken as evidence excluding this process. The oxidation of sulfide and/or the rapid precipitation of dissolved hydrogen sulfide as metal sulfides can modulate pool sizes such that increases in sulfide concentration may be undetectable. Direct measurements of sulfate reduction rates using ³⁵SO₄²⁻ as a tracer can overcome these limitations, but the currently used techniques are time-consuming and require considerable effort. We have adapted these methods and developed a simpler and less time-consuming procedure.

Current radiotracer methodology for determination of sulfate reduction rates involves the incubation of ³⁵SO₄²⁻ with sediments followed by extraction and quantitation of the produced ³⁵S-sulfides including H₂³⁵S, H³⁵S⁻, Fe³⁵S, and Fe³⁵S₂. It has recently been shown that isotopic exchange between some of these compounds and S⁰ occurs, thereby limiting the usefulness of radiotracers in speciation studies (13). Therefore, a single-step method has been proposed in which

the pool of total reduced inorganic sulfur (TRIS) is reacted with a solution of Cr(II) and HCl (12). This procedure involves the reduction of TRIS (to H₂S), but not organic S or most sulfuroxy anions (7, 12), by heating samples and the HCl-Cr(II) extractants during a reflux distillation. Liberated sulfide is then flushed through the reflux condenser into a sulfide-trapping solution (5 to 10% zinc acetate). These distillations must be carefully monitored and are extremely time-consuming when the analysis includes multiple samples. We have adapted the above single-step chromium reduction method for the estimation of sulfate reduction rates and inorganic sulfur pools in large numbers of samples.

The adapted method involves the extraction of sulfides from samples placed in 120-ml serum bottles. The serum bottles contained an anoxic headspace and a sulfide trap (Fig. 1). All reagents, traps, and samples must also be anoxic to prevent the premature oxidation of the Cr(II) solution. Sample manipulations were conducted in a well-working anaerobic glove bag.

Sediment or water samples can be preserved by being mixed with an anoxic solution of 20% zinc acetate (boiled and cooled under N₂; ~2 ml · g of wet sediment⁻¹) and stored at -20°C. For analysis, these samples (1 to 2 g) or iron sulfide minerals were added directly to the serum bottles while inside the anaerobic glove bag. Alternatively, they can be transferred by pipette or injected via syringe into serum bottles. To prevent excessive pressure in the serum bottles due to CO₂ or H₂S volatilization, sample sizes should be kept to a minimum; normally <2 g of sediment in the recommended serum bottles is appropriate. Test tubes (12 by 75 mm) containing 2.5 ml of anoxic 10% zinc acetate solution were placed inside the serum bottles as sulfide traps. The serum bottles were fitted with 1-cm-thick rubber stoppers (Bellco Glass Inc., Vineland, N.J.) and removed from the glove bag. If a glove bag is not available, these reagents can be added to the serum bottles under a steady stream of O₂-free N₂ by using the Hungate technique (14). Water samples or slurries consisting of fine-grained sediments can also be injected directly into serum bottles without prior fixation of free hydrogen sulfide with zinc acetate. A 1 M Cr(II)-HCl solution (8 ml) (12) followed by anoxic 12 N HCl (4 ml) is then injected into the serum bottles through the stoppers. The latter reagent may be prepared by heating 160-ml serum bottles containing 100 ml of HCl in a water bath (~90°C) while continuously flushing the reagent with N₂ for

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FIG. 1. Schematic representation of the passive extraction procedure used for the determination of sulfate reduction activity or TRIS in sediment or water samples. A stoppered 120-ml serum bottle contains the Cr(II) solution and the sediment sample. A test tube (12 by 75 mm) containing 2.5 ml of 10% zinc acetate is used to trap the liberated H_2S .

approximately 40 min. The acid fumes from this procedure can be vented through a bicarbonate solution. The bottles are then removed from the hot water bath and stoppered. The HCl and Cr(II) solutions can be stored for several months inside stoppered serum bottles under a N_2 headspace. After addition of the Cr(II) and HCl reagents, serum bottles are placed on a rotary shaker (~ 150 rpm) for the duration of the extraction. Oxidized samples or samples which contain high concentrations of reduced S may require additional chromium solution. Such cases are evident as the chromium-HCl solution becomes oxidized and changes from a dark blue to a bright green color. The trapping solution precipitates the liberated H_2S as ZnS . After 30 h or as indicated, the sulfide traps were removed from the serum bottles. A homogeneous ZnS suspension was ensured by vigorously vortexing the traps followed by treatment with a sonicating water bath for about 30 s. Sulfide was quan-

tified in aliquots of this suspension by using the methylene blue assay (8) or by scintillation counting (10 to 20% [vol/vol] in Ultima-Flo AF scintillation fluid; Packard Instrument Co., Downers Grove, Ill.).

We have evaluated the time required to extract dissolved sulfide and sulfur from pure (S-containing) minerals. At the same time we determined the trapping efficiency for these species (Fig. 2). Recovery of Na_2S was complete within 2.5 h, while comparable recoveries of iron monosulfide (FeS) and pyrite (FeS_2) required 10 and 20 h, respectively (Fig. 2A). Only partial recovery of particulate S^0 was observed with extractions as long as 7 days (Fig. 2B), but S^0 dissolved in acetone was rapidly and completely recovered. Additional experiments indicated that sulfide recovery was linear and complete for concentrations ranging from 1 to 20 mg of S (data not shown), above which the recovery decreased. Of course, other forms of S exist in the environment (4, 15) and were not specifically evaluated in our experiments. For instance, polysulfide is commonly found in porewaters of marine sediments (6). Acidification causes polysulfide to decompose to H_2S and S^0 (12), and the latter will decrease the efficiency of polysulfide recovery.

The applicability of the passive extraction technique for the recovery of sulfides from sediments was evaluated by determining TRIS over time in samples from a landfill-leachate-impacted aquifer (Fig. 3). TRIS recovery from the samples amended with known quantities of pyrite was equivalent to the sum of TRIS recoveries from sediment and pyrite alone. Thus, the sediment had no influence on the extraction of the added pyrite. The additional time required to recover S from the sediment samples relative to pyrite may be due to the fact that these sulfides are more resistant to chromium and acid extraction than pure S-containing minerals. Thus, an extended extraction time (~ 70 h) may be necessary for the complete recovery of TRIS in some samples.

In a comparison of the passive extraction technique (30 h) and the distillation method previously described by Fossing and Jørgensen (12), sulfate-reducing activity and TRIS pools in two different anaerobic sediment samples were evaluated (Table 1). The two methods yielded virtually identical results for

FIG. 2. Sulfur recovery as a function of time (< 20 h [A] or > 20 h [B]) by using the passive extraction procedure with 1 mg of sulfur as either Na_2S , powdered elemental sulfur (S^0), or S^0 dissolved in acetone and 5 mg of sulfur as iron monosulfide (FeS) or pyrite (FeS_2). FeS , FeS_2 (99.99% pure; Alfa AESAR, Ward Hill, Mass.), and elemental sulfur (Sigma Chemical Co., St. Louis, Mo.) were added as dry powders. Dissolved S^0 was prepared by dissolving excess particulate S^0 in 100 ml of acetone. The solution was filtered to remove undissolved S^0 . $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (Aldrich Chemical Co.) stock solutions were prepared with anoxic 0.01 N NaOH. Each point represents the mean of duplicate extractions with the standard error (error bar) given where the value exceeds the dimension of the symbol.

FIG. 3. The recovery of TRIS over time for 1 g of landfill-leachate-impacted aquifer sediment, 1 mg of FeS_2 , or a mixture of 1 g of sediment and 1 mg of FeS_2 . The values represent the means of duplicate extractions with the standard errors (error bars) given where values exceed the dimensions of the symbols.

both the highly active brackish sediments from San Diego bay (19) and the moderately active sediments from a landfill-leachate-impacted aquifer (5). While such results are promising, we recognize that they are far from exhaustive and that samples from multiple environments will have to be similarly evaluated. It remains to be seen whether our findings will prove to be generalizable.

It is important to point out that we consider the rates of sulfate reduction based on the passive extraction method as estimates only. This is because sulfate reduction rates could be underestimated in some samples where S^0 may represent an important portion of the reduced sulfur pool. S^0 may be formed by the oxidation of reduced S species. It is also known that radioisotopic evaluations of sulfate reduction rates may be biased by exchange reactions between the S^0 pool and H_2^{35}S formed by the microbial reduction of $^{35}\text{SO}_4^{2-}$ (1, 13). The extraction of nondissolved S^0 is incomplete by chromium reduction techniques, but it is more reliably extracted with the heating and reflux distillation procedure (12) compared to our proposed passive modifications. Other techniques, such as solvent extractions, have been recommended for circumventing

the problems associated with the poor recovery of S^0 in sulfate reduction assays (1, 12).

Additional experiments indicated that the passive extraction technique could be used for the speciation of sulfides. Following the addition of 2 N HCl, the acid-volatile sulfides (H_2S and FeS) were completely recovered from a mixture of pure sulfur-containing minerals (data not shown). Pyrite was not extracted under these conditions over a 30-h period but was recovered following the addition of the reduced chromium solution and 12 N HCl.

In our hands, both chromium reduction techniques gave comparable results. However, the passive extraction method has several important advantages over the distillation procedure (12). First, since it is not necessary to monitor the extractions during sulfide distillation, a large number of samples (over 100 per day) can be processed over a relatively short time. Furthermore, unlike the reflux extractions, carryover of unreacted $^{35}\text{SO}_4^{2-}$ into sulfide traps does not occur when the passive technique is used. Thus, it is not necessary to wash unreacted $^{35}\text{SO}_4^{2-}$ from samples prior to the sulfide extraction step, and time and labor are saved. We have used the modified technique to monitor low levels of sulfate reduction in biodegradation experiments, to estimate sulfate reduction rates in natural samples, and to measure reduced S pools in samples from both contaminated and pristine aquifers. In our hands, the passive extraction method has allowed the relatively rapid processing of thousands of samples which would otherwise require full-time personnel and dedicated equipment use.

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TABLE 1. Comparison of techniques used for the estimation of sulfate reduction rates and the extraction of TRIS

Sediment type	Sulfate reduction rate ($\text{nmol} \cdot \text{g}^{-1} \cdot \text{day}^{-1}$) ^a		TRIS pool ($\mu\text{mol S} \cdot \text{g}^{-1}$)	
	Reflux	Passive	Reflux	Passive
Aquifer	1.1 (0.001)	1.1 (0.002)	3.8 (0.85)	4.3 (0.72)
Marine	115.5 (0.32)	116.1 (1.07)	85.2 (19.90)	83.5 (23.91)

^a Sediments were amended with 2 μCi of carrier-free $\text{Na}^{35}\text{SO}_4 \cdot \text{g}$ of wet sediment⁻¹ and incubated in serum bottles under a 100% N_2 headspace for 48 h in the dark. Bottles were opened at the end of the incubation, and weighed aliquots (1 g) of sediment were fixed in 2 ml of 10% anoxic zinc acetate. TRIS was extracted from replicate fixed sediment samples by the reflux condenser method or the method described in this paper. Sulfate reduction rates were determined by calculating the fraction of the sulfate pool which was reduced during the incubation (7). Sulfate reduction rates and TRIS determinations represent the means of three and five determinations, respectively; standard deviations are shown in parentheses.

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