

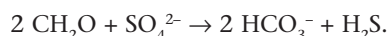
A cold chromium distillation procedure for radiolabeled sulfide applied to sulfate reduction measurements

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Abstract

The separation and detection of extremely small amounts of radiolabeled reduced sulfur species for the determination of very low sulfate reduction rates (SRR) with the $^{35}\text{SO}_4^{2-}$ radiotracer was improved by optimization of the entire distillation and detection process. By reducing the amount of background radioactivity, the threshold from which turnover of radiotracer can be detected was lowered considerably. Reduction of the background radioactivity was achieved by (1) reducing cross-contamination between distillations by modifying the distillation setup, and (2) preventing an unidentified ^{35}S -containing compound that greatly contributes to the background from reaching the final trap by lowering the distillation temperature. These improvements allow the measurement of low SRR, shorter incubation times, and the use of less radiotracer. Experiments with pure sulfur minerals and a variety of sediments verified that the efficiency of the new method is equal to the hot single-step chromium reduction method, but with a greatly improved reproducibility.

Dissimilatory sulfate reduction (DSR) is a key process in the anaerobic degradation of organic matter in marine sediments. The overall reaction can be written as



Sulfate-reducing bacteria reduce sulfate to sulfide, which either remains in solution as hydrogen sulfide or precipitates as various forms of metal (mostly iron), mono- and disulfides, or elemental sulfur (ES). These combined inorganic end products of sulfate reduction are termed total reduced inorganic sulfur (TRIS). The importance of DSR as a terminal electron acceptor process in marine sediments has been established through measurements of sulfate reduction rates (SRR) by using carrier-free radiolabeled $^{35}\text{SO}_4^{2-}$ as a tracer. The volume of tracer that is added is in the range of 2 to 10 μL and contains only negligible amounts of sulfate, ca. 1 nmol sulfate per injection of ca. 50 kBq $^{35}\text{SO}_4^{2-}$. Therefore no change of porewater sulfate concentration occurs. Radiotracer incubations

generally proceed by injecting carrier-free $^{35}\text{SO}_4^{2-}$ radiotracer into the undisturbed sediment followed by incubation for hours to days. Incubations may be terminated by freezing or transferring the radiolabeled sediment into 20% (w/v) zinc acetate solution. By comparing the activity of the radiolabeled TRIS to the total sulfate radiotracer a reduction rate can be calculated as shown below in Eq. 1.

$$\text{SRR} = [\text{SO}_4] \times P_{\text{SED}} \times \frac{a_{\text{TRIS}}}{a_{\text{TOT}}} \times \frac{1}{t} \times 1.06 \times 1000 \quad (1)$$

where SRR is the sulfate reduction rate ($\text{nmol cm}^{-3} \text{d}^{-1}$); $[\text{SO}_4]$ is the sulfate concentration in the porewater of the sediment sample (mmol L^{-1}); P_{SED} is the porosity of the sediment ($\text{mL porewater cm}^{-3} \text{sediment}$); a_{TRIS} is radioactivity of TRIS (counts per minute [cpm] or decays per minute [dpm]); a_{TOT} is total radioactivity used (cpm or dpm); t is incubation time in days; 1.06 is the correction factor for the expected isotopic fractionation (Jørgensen and Fenchel 1974); 1000 is the factor for the change of units from mmol L^{-1} to nmol cm^{-3} .

The detection limit of the radiotracer method for measuring SRR depends on the efficient separation of a minute amount of radiolabeled reduced sulfur (a_{TRIS}) from an overwhelming background of unreacted $^{35}\text{SO}_4^{2-}$ radiotracer. If, for example, the SRR is $1 \text{ pmol cm}^{-3} \text{d}^{-1}$, the porewater concentration is 10 mM SO_4^{2-} , the porosity 0.8, incubation time is 10 d, and 1 MBq of tracer is added, then the amount of TRIS produced is only about 1.2 Bq. This would generate a radioactive

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count rate of 71 cpm above background, assuming distillation and counter efficiencies of 100%.

The vast majority of SRR measurements in the marine environment have been restricted to the upper centimeters to meters of the seafloor with high turnover rates of 1 to 200 nmol cm⁻³ d⁻¹ (e.g., Martens and Klump 1984; Albert 1985; Jørgensen et al. 1990; Ferdelman et al. 1999; Schubert et al. 2000; Jørgensen et al. 2001). However, in deeper and less active sediments, where the rates are expectedly lower by several orders of magnitude, the radiotracer method may have insufficient sensitivity (Fossing et al. 2000; Parkes et al. 2000).

Three factors can, in theory, be manipulated independently to increase the sensitivity of SRR measurements: incubation time (*t*), the total amount of radioactivity (*a*_{TOT}), and the detection limit of *a*_{TRIS}. With an increase in either incubation time (*t*) or *a*_{TOT}, *a*_{TRIS} proportionally increases. Incubation time can be increased but not indefinitely. For the limiting case where the consumption of total tracer is negligible (i.e., a tracer experiment), a theoretical maximum incubation time, due to the decay of the ³⁵S radiotracer, is reached:

$$t_{\text{MAX}} = 1/(\ln 2 \times t_{1/2}) \quad (2)$$

where *t*_{MAX} is the maximum incubation time beyond which the decay of labeled TRIS becomes faster than the production of newly labeled TRIS, and *t*_{1/2} the half-life of the isotope. The maximum incubation time is independent of the rate of sulfate reduction and is controlled by the half-life of the isotope. The half-life time of ³⁵S is 88 d, which defines a maximum for ³⁵SO₄ incubations of 127 d. Furthermore, long incubation times are not necessarily desirable. The prokaryotic community, the sediment chemistry, and the turnover rates may change with time from the in situ state. To obtain representative rate measurements, all parameters influencing the bacterial community in the sediment should be kept constant over the entire incubation time. Moreover, the radioactivity of the reduced sulfur compounds must also increase linearly with incubation time to be able to calculate a true sulfate reduction rate. For instance, if the reduced sulfur pool is very small and turns over rapidly, it will quickly saturate with ³⁵S and lose its capacity to record the increase in TRI³⁵S over time. Thus a reliable SRR cannot be estimated (Moeslund et al. 1994; Fossing 1995).

Increasing the amount of tracer creates another problem. We have observed that ³⁵SO₄²⁻ obtained from different manufacturers (Risø, Amersham) contain a compound that contributes to the background, i.e., the amount of radioactivity that is found in the TRIS fraction without being produced by DSR. The background associated with the tracer itself increases proportionally with the amount of tracer added. Furthermore, an increase in the amount of tracer (*a*_{TOT}) has practical limitations in matters of cost and safety. The amount of sample that can be efficiently processed is also a limiting factor. Upscaling of the entire distillation setup reaches practical limits very soon. The counting efficiency for ³⁵S by modern scintillation

counters is close to 95%, leaving little space here for improvement. The most promising way to increase the sensitivity of the method is to lower the detection limit of TRI³⁵S by lowering of the background.

Background and blanks—When a distilled sample is counted on a scintillation counter, background is an inherent part of the total number of counts as shown in Eq. 3:

$$a_s = a_{\text{TRIS}} + B_s \quad (3)$$

where *a*_s is the measured amount of radioactivity in the distilled sample, *a*_{TRIS} is the activity due to the TRI³⁵S formed during DSR, and *B*_s is the total sample background that is attributable to all background sources. (In this discussion and hereafter we use lower-case letters to denote a measured value and upper-case letters to denote the measurand, or quantity that we want to determine.)

The sample background, *B*_s, comprises three components:

$$B_s = B_c + B_D + B_T \quad (4)$$

where *B*_c, or counter background, is the count rate inherent to the environment and scintillation fluid without addition of a radioactive sample; *B*_D, or distillation background, is the background radioactivity resulting from the distillation equipment itself (e.g., traces of radioactivity in gas lines); and *B*_T, or tracer background, is the count rate attributed to the ³⁵S radio-labeling and distillation but not arising from bacterial sulfate reduction.

We are interested in obtaining a well-constrained estimate of the background component (*B*_s) of our distilled radioactivity *a*_s. To estimate *B*_s, or any of the individual sources of background radioactivity contributing to the overall background, we must make a series of blank measurements. The counter blank, *b*_c, is equivalent to the number of cpm that are recorded by the scintillation counter when a nonradioactive sample is measured. The nonradioactive sample contains the same amounts of scintillation fluid and ZnAc as a normal sample. The counter blank, *b*_c, is independent of the method of distillation and is equal to *B*_c. It might be lowered slightly by enforced cleanliness of the scintillation counter and thorough evaluation of the counting conditions. The distillation blank, *b*_D, which equals (*B*_c + *B*_D), takes into account background radioactivity in the distillation apparatus and reagents. Most of this distillation background radioactivity, *B*_D, arises because of memory effects (carry-over) between distillations. The distillation blank, *b*_D, can be determined by distilling a nonradioactive sample immediately after a radioactive sample has been treated in the distillation equipment, followed by the same counting procedure as for radioactive samples.

The sample blank (*b*_s) gives the total number of counts that are not associated with sulfate reduction and is equivalent to *B*_s (Eq. 5):

$$b_s = B_s = B_c + B_D + B_T \quad (5)$$

To determine b_s a sediment sample is first vigorously mixed with 20% (w/v) ZnAc to stop sulfate reduction and after 30 min radiolabeled $^{35}\text{SO}_4^{2-}$ is added in the same amount as used for sulfate reduction measurements. Because bacterial activity is stopped before the addition of the tracer, no DSR should take place and, accordingly, no radiolabeled TRIS should form. The number of counts found in the trap should be equal to $B_C + B_D$. However, the tracer background, B_T , additionally contributes to B_S . Thus, B_S represents the sum of counter, distillation, and tracer backgrounds ($B_S = B_C + B_D + B_T$) and can be estimated from the sample blank, b_s . It is important to note that we only ever estimate B_C , B_S , or ($B_C + B_D$) by measuring b_C , b_S , and b_D . There are no means of directly ascertaining B_D or B_T alone. As $b_s = B_S$, $b_C = B_C$, and $b_D = (B_C + B_D)$, we can calculate $B_D = (B_C + B_D) - B_C$ and $B_T = B_S - (B_C + B_D)$, respectively.

The Cr-II method—Over the last decades, several methods have been developed to separate the reduced sulfur species from sediments. Sorokin (1962) first introduced direct radiotracer measurements of sulfate reduction rates. Most separations are based on the methods of Zhabina and Volkov (1978) who introduced the hot acidic chromous chloride distillation procedure for separating reduced sulfur species comprising hydrogen sulfide, ferrous sulfide, pyrite, and elemental sulfur. The extracted sulfur species were liberated as hydrogen sulfide and trapped in cadmium acetate solution as solid cadmium sulfide. Subsequent studies have modified this technique according to their needs. Based on the assumption that all H_2S produced by bacteria was retained in the sediment as free or acid volatile sulfur (AVS) (i.e., hydrogen sulfide and ferrous sulfide), several authors acidified the sediment in order to retrieve all bacterially produced hydrogen sulfide (Jørgensen 1978 and references therein). Howarth and coworkers (e.g., Howarth and Gibling 1983) showed that the bacterially produced H_2S also reacts to form pyrite and other non-AVS phases. They concluded that radiotracer measurements solely based on AVS determinations might underestimate the true SRR.

Based on the extraction scheme of Zhabina and Volkov (1978), Canfield et al. (1986) and Fossing and Jørgensen (1989) developed a single-step method that retrieves all reduced inorganic sulfur species by boiling the sample in acidic chromous chloride solution. The chromium reducible sulfur (CRS) includes pyrite and ES. AVS can either be extracted in a step prior to CRS extraction by acidifying the sample followed by degassing or together with the CRS when the sample is boiled in hot acidic chromous chloride solution. The reason for heating the sediment is the very stable S-S bond in ES. Only at elevated temperatures is the Cr^{2+} able to transfer an electron to one of the S atoms, thereby reducing the strength of the S-S bond (Luther 1987). Fossing and Jørgensen (1989) showed that, even with boiling, the recovery of ES depends on its degree of crystallinity. Whereas ES dissolved in acetone is recovered almost entirely even with cold chromium

solution ($91.4\% \pm 2.8\%$), recovery drops close to zero for granular and colloidal ES.

The method of Fossing and Jørgensen (1989) has proven to be robust, albeit fairly labor-intensive. Nonetheless, when using the hot chromium distillation, even under stringently clean conditions, a minute amount of radioactivity distills over and creates a signal that cannot be attributed to DSR. This background problem is inherent to the hot chromium distillation. The sample background, B_S , can be lowered by washing the sample several times prior to the distillation to remove as much $^{35}\text{SO}_4^{2-}$ as possible. As long as SRR are high and produce enough radiolabeled TRIS to obtain a ^{35}S -TRIS signal well above B_S , this problem can be neglected. However, as soon as a_{TRIS} is low (<100 cpm), it becomes crucial to keep B_S even lower to detect the actual signal. The sample background, B_S , appears to be a function of reaction temperature and acid strength. Whereas acid concentration can be regulated to minimize B_S , heat is required to make the measurement fully quantitative. Eliminating heating would improve the method as long as all TRIS is quantitatively distilled.

Several studies have used passive distillation procedures by which the sample and all reagents were mixed in a container with a H_2S trap hanging in the gas phase above the solution to trap all produced sulfides. The passive distillation procedures work at cold or at sub-boiling temperatures, are less labor intensive, and allow the simultaneous processing of a larger number of samples. However, the passive distillation methods tend to suffer from low recovery (Howarth and Gibling 1983), especially for ES (Ulrich et al. 1997). Although ES may not form a significant fraction of the total TRIS pool, a significant fraction of the radioactively labeled sulfide may still end up in this fraction during an SRR experiment.

To improve the recovery of ES, Hsieh and Yang (1989) used N, N-dimethylformamide (DMF). Not only does DMF solubilize ES rapidly, but DMF is also an organic solvent commonly used to accelerate chemical reactions. This reagent destabilizes the S-S bonds and, therefore, allows the chromium to reduce it to sulfide (Hsieh and Yang 1989). In theory, a carryover of nonreacted sulfate is more limited or even absent by this procedure due to the lower distillation temperatures. The passive distillation has certain practical disadvantages for the recovery of very low levels of radiotracer-labeled sulfide. Because the trap hangs inside the reaction flask, aerosols containing radiolabeled $^{35}\text{SO}_4^{2-}$ might reach the trap, carrying over nonreacted sulfate and thereby increasing B_S . Moreover, the trapping vial may be contaminated on the outside, requiring either extremely thorough cleaning or transfer of the trapping solution into a new vial. CO_2 produced during the acid dissolution of carbonate-containing samples creates high pressure and can cause the reaction flask to leak or burst. Sample size either has to be kept to a minimum, a step to remove the carbonates is required prior to distillation, or a pressure-compensation has to be installed. Therefore, we chose to combine the DMF method of Hsieh and Yang (1989) with an active distillation at room temperature.

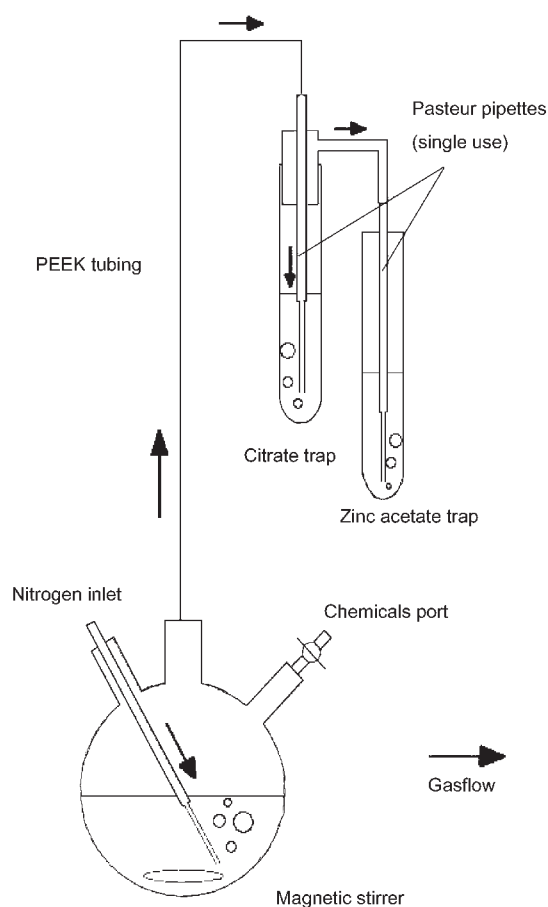


Fig. 1. Schematic view of the cold distillation apparatus. See text for description.

Methods and procedures

Hot distillation—In the hot single-step chromium reduction method of Fossing and Jørgensen (1989), a sample containing sediment and zinc acetate (ZnAc) is centrifuged and the supernatant removed. Two to three grams of sediment are mixed with 20 mL of 50% (v/v) ethanol-water solution and placed into a 3-neck round bottom flask that is connected to a reflux cooler. Nitrogen is introduced through one of the necks; the other neck is used as a chemical port. The apparatus is gassed with N_2 for at least 10 min to remove any O_2 . For a single-step distillation 8 mL of 6N HCl and 16 mL of a 1 M $CrCl_2$ solution are injected through the chemical port and the slurry is boiled for 40 min. The produced volatile H_2S is bubbled through a disposable Pasteur pipette into a trap filled with 7 mL of 5% (w/v) ZnAc-solution to trap all sulfide as zinc sulfide. To prevent the trap from overflowing, a drop of antifoam is added. In cases where samples contain only little sulfide, some carrier (zinc sulfide suspension or sodium thiosulfate solution) is added to enhance the efficiency of the method; we typically employ 500 μL of a 50 mM ZnS suspension.

Cold distillation—Fig. 1 shows the apparatus for the cold distillation. The setup is similar to the hot distillation but with the following important modifications: (1) The reflux cooler is replaced by PEEK (Poly-Ether-Ether-Ketone) tubing (1/8-inch outer diameter). PEEK is gas impermeable and nonreactive. It can be cleaned easily by flushing with water. (2) Between the PEEK tubing and the zinc acetate trap another trap with 7 mL of a 0.1 M citrate solution (19.3 g citric acid, 4 g NaOH in 1 L H_2O , pH 4) is placed. This trap is necessary to prevent any aerosols from reaching the final trap. In the hot distillation, this trap is not necessary as condensed water runs down the reflux cooler, stripping the aerosols from the upward flowing gas stream. Experience has shown that the citrate trap does not have to be replaced after every distillation. All connections between the glassware and the PEEK tubing are made from short pieces of Viton tubing, mechanically secured with silicone tubing placed over it.

The sample is transferred to a 3-neck round-bottom glass flask 20 mL DMF is added together with a magnetic stir bar to secure an efficient mechanical breakup of the sample. Whereas DMF enhances the reactivity of reduced sulfur species, it also allows oxidation to take place at an enhanced rate. To prevent oxidation prior to distillation, extended contact of the sediment-DMF slurry with air should be avoided. As soon as the sample is mixed with DMF, the reaction flask is connected to the gas line and flushed with N_2 for 10 min. Subsequently, 8 mL 6 N HCl are injected through the chemical port, followed by 16 mL 1 M $CrCl_2$ solution. The sample is then bubbled at a rate of 2 to 5 bubbles per second with N_2 for 2 h and continuously stirred. Over the last 15 min, the gas flow rate is increased to remove the last vestiges of sulfide from the system. If the sample is rich in carbonate, the acid has to be added slowly to avoid heavy foaming during the liberation of CO_2 . If the HCl concentration is too low, and consequently the pH too high, the Cr(II) solution will appear brick red in color. With additional HCl, the Cr(II) solution will revert to its characteristic blue color. Similar to the hot method, the liberated sulfide is trapped as zinc sulfide in 7 mL of 5% (w/v) ZnAc-solution with a drop of antifoam. Like for the hot method, a sulfide carrier should be used for low-sulfide samples.

Efficiency control experiments—To test the efficiency of the new method, pure mineral sulfur phases were prepared and then distilled using both the hot and cold chromium reduction method. The quantity of trapped sulfide was determined according to the method of Cline (1969).

The following minerals were prepared and investigated:

BaSO₄. One liter of 0.25 M barium hydroxide solution (47.34 g $Ba[OH]_2 \times H_2O$ in 1 L H_2O , pH adjusted to 7 with HCl) was mixed with 200 mL of 1.25 M sodium sulfate solution (177.55 g Na_2SO_4 in 1 L H_2O). The precipitated $BaSO_4$ was washed three times in deionized water and dried at 60°C.

Dissolved sulfur. An exact amount of flowers of sulfur (10 to 100 mg) was dissolved in 20 mL of acetone.

Crystalline sulfur. Flowers of sulfur (10 to 100 mg) were directly weighed into the reaction flasks.

FeS. 200 mL of 0.6 M ferrous sulfate solution (167 g $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ in 1 L H_2O) was mixed with 200 mL of 0.6 M sodium sulfide solution (144 g $\text{Na}_2\text{S} \times 9\text{H}_2\text{O}$ in 1 L H_2O). The resulting precipitate was washed five times in de-ionized water to remove free sulfide.

Natural FeS_2 . Originating from an ore-processing flotation plant (Schippers and Jørgensen 2001). The material was ground to 50- to 100- μm grain size.

Synthetic FeS_2 . The mineral was prepared according to Fossing and Jørgensen (1989). It was cleaned of adhering elemental S by mixing several hundred milligrams of material with 250 mL n-hexane in a stoppered glass bottle and placing it on a shaker overnight. The remaining grains were rinsed in acetone. The ES that was dissolved in acetone could be precipitated as colloidal sulfur upon addition of water. Extraction was complete when precipitation could no longer be observed in the washing solution.

Background and tracer experiments—The tracer itself contains a component, B_p , that can be distilled and thus contributes to the sample background, B_s . To investigate this radioactive contaminant, we separated the tracer using ion chromatography (pump: Waters 510; column: Waters IC-Pak Anion 4.6 mm \times 5 cm; detector: Waters 430 Conductivity detector; eluent: 1 mM isophthalic acid; flow: 1.0 mL min^{-1}). The retention time for sulfate was about 11 min. The outflow from the detector was collected using a fraction collector (six time intervals: 0-2, 2-6, 6-12, 12-18, 18-24, 24-40 min). The six fractions were distilled using the hot chromium reduction method of Fossing and Jørgensen (1989). The amount of radioactivity in each fraction prior to and after distillation was compared. In an identical experiment the outflow of the detector was collected in 2-min time-intervals and counted. The set of samples from the latter experiment was kept and counted bimonthly over a period of 2 y to establish whether the $^{35}\text{SO}_4^{2-}$ tracer contained any other radioisotope than ^{35}S as judged from the ^{35}S half-life of 88 d.

Scintillation counting—Radioactivity was determined by liquid scintillation counting (Packard 2500 TR) with a counting window of 4 to 167 keV, no luminescence correction, and high sensitivity mode turned off. We observed that this last feature increased background variability of count rates without a meaningful gain in sensitivity or lowered detection limit. A cut-off of the low energy range (0 to 4 keV) eliminates a large fraction of low energy background counts, while only minimally reducing the count rate of ^{35}S , whose β -energy spectrum lies at higher energies (up to 167 keV). All activities presented in this study were recorded in the range of 4 to 167 keV. Counting time was 10 min for all samples. The scintillation cocktail used was Lumasafe Plus[®] (Lumac BV, Holland) mixed with the trapped ZnS 2:1, vol:vol. Results are given as cpm.

Preparation of radiolabeled sediments—Sediments from various sites were obtained for testing the cold distillation procedure. The types of sediments used comprise a variety of marine environments with respect to sediment type, salinity, sulfate reduction rate, and organic carbon content. ^{35}S radio-

labeled slurries were prepared using several hundred grams of sediment incubated with 10 MBq $^{35}\text{SO}_4^{2-}$ radiotracer in gas-tight plastic bags (Hansen et al. 2000) for 3 to 4 d. Subsequently the mud was transferred and mixed into an equivalent amount of 20% (w/v) ZnAc-solution to terminate the incubation. The homogenized slurries were dispensed with a pipette and distilled using both the hot and cold distillation methods. The distillation time necessary to obtain reproducible results with the cold chromium distillation was determined from a time-course experiment. Hence, after the distillation was initiated the ZnAc-trap was changed after 30, 60, 90, 120, 150, 210, and 300 min and the amount of radioactivity in each trap was measured. The sediments used for the time series and the efficiency control experiments were as follows:

Namibia upwelling. The sediment (22°38'51" S, 4°18'25" E at a water depth of 70 m) is diatomaceous ooze deposited within the oxygen minimum zone. Sulfate reduction rates are extremely high and range from 100 to 2000 $\text{nmol cm}^{-3} \text{d}^{-1}$. Makran Plateau off Pakistan. The sample consists of distinctly laminated olive gray mud (A. Lückge, pers. comm. unref.). It was obtained during the RV *Sonne* cruise SO 130 at 22°56'34" N, 66°38'77" E at 831 m water depth under the oxygen minimum zone. Sulfate reduction rates were expected to be extremely low.

Peru. The sample was taken during the RV *Sonne* cruise SO 147 in summer 2000 in the coastal upwelling off Peru within the oxygen-minimum zone. Sulfate reduction rates are high, between 50 and 700 $\text{nmol cm}^{-3} \text{d}^{-1}$ at the surface. The sediment is sandy clay.

Weddewarden. The sampling site is located in the intertidal zone of the estuary of the River Weser in northern Germany. Salinity ranges from 4‰ to 20‰, temperature from 3°C to 30°C. The sediment consists mostly of silt with ca. 10% fine sand and 10% clay. SRR are highly variable and range from 1 to 100 $\text{nmol cm}^{-3} \text{d}^{-1}$.

Sample blank (B_s). Makran Plateau sediment (Mak) was preserved in 20% (w/v) ZnAc-solution to stop sulfate reduction prior to addition of $^{35}\text{SO}_4^{2-}$.

Assessment

Specificity toward S phases—To test the specificity of the cold distillation procedure, we conducted control experiments with different pure sulfur phases. The results show that the cold chromium distillation technique produces results comparable to the hot distillation (Table 1). The only result that differs significantly from 100% recovery is that for crystalline sulfur. With the cold distillation we extracted $74.8\% \pm 7.6\%$ of crystalline sulfur whereas the recovery was only $10.4\% \pm 3.8\%$ with the hot method. Barite was not reduced during distillation by either method. For FeS both methods fell slightly short of 100%, probably caused by oxidation of FeS during preparation and storage or inhomogeneities in the suspension. For natural mineral FeS_2 , the cold method recovered $88.4\% \pm 3.5\%$, slightly less than Canfield et al. (1986) found in their study ($95\% \pm 2.6\%$). The

Table 1. Recovery of pure sulfur phases by hot and cold chromium reduction methods

Mineral	Recovery, %	SD*
Cold		
Barite	0.014	0.005
S crystal	74.80	7.61
S dissolved	90.04	1.45
FeS	95.88	9.72
Pyrite crystals (50 to 100 μm)	88.40	4.00
Synthetic pyrite	101.28	5.62
Hot		
Barite	0.005	0.001
S crystal	10.35	3.82
S dissolved	84.47	5.42
FeS	98.36	2.10
Natural pyrite†	95.00	2.60
Synthetic pyrite‡	100.00	8.40

*SD, standard deviation.

†Data taken from Canfield et al. (1986).

‡Data taken from Fossing and Jørgensen (1989).

synthetic pyrite was completely extracted within 2 h (recovery $101.3\% \pm 5.6\%$), which is consistent with the results of Fossing and Jørgensen (1989) ($100\% \pm 8.4\%$). The age of the sediment and thereby the crystallinity of the mineral phases influences the distillation time and the specificity of the method. For the rather young sediments in our study we found 2 h to be sufficient to obtain results comparable with the hot distillation

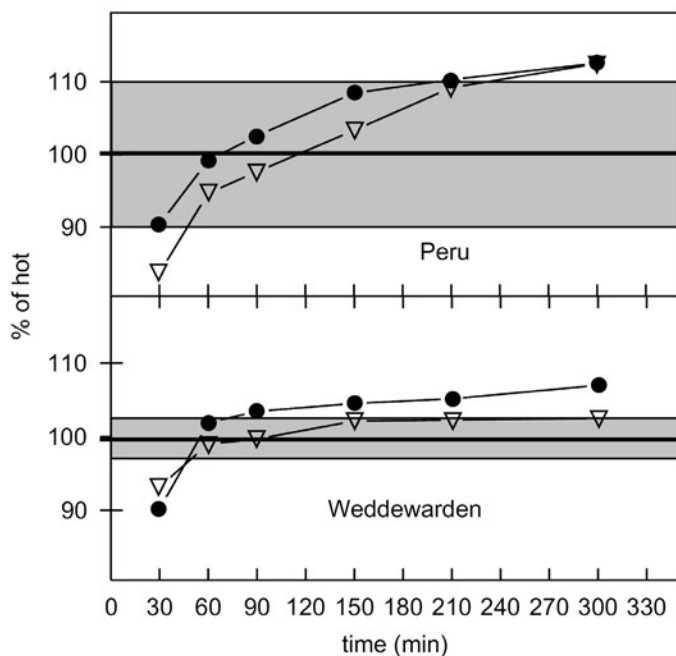


Fig. 2. Time-course experiment for cold distillation, shown for two different sediments. The results are expressed as percent of the average of the hot distillation. The gray area is one standard deviation of the hot method.

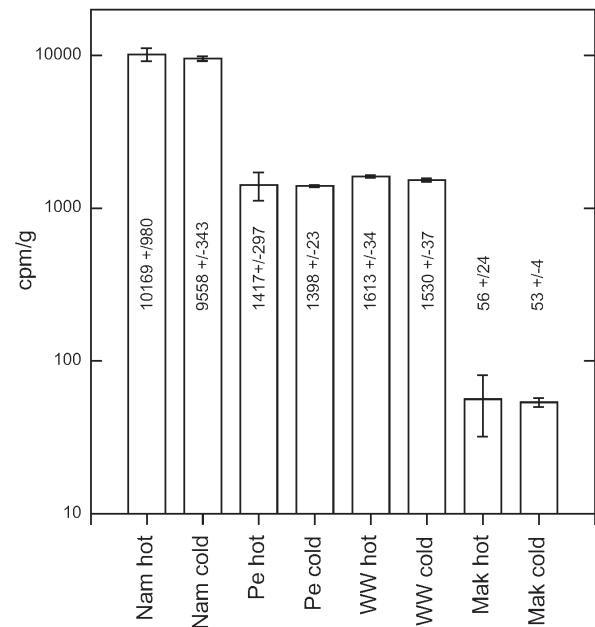


Fig. 3. Measured a_{TRIS} (cpm g^{-1}) for sulfate reduction experiments with various sediments, using the hot single-step chromium reduction method and the cold distillation method. The error bars are one standard deviation ($n = 6$).

(Fig. 2). We found that it is crucial to keep the sample as fine-grained as possible and in constant suspension, otherwise the reaction is slowed considerably.

Dissolved ES is extracted with almost equal efficiency by both methods, but for the hot method the recovery is low for crystalline ES (Table 1). Because DMF is added during the cold method, ES is rapidly brought into solution, which allows the chromium to reduce it. This is crucial for the efficiency of the cold method. Although ES concentrations in sediment may be low, a significant fraction of the ^{35}S tracer may end up in this pool through oxidative processes or isotope exchange reaction (Fossing 1995; Fossing et al. 2000).

Efficiency of radioactive TRIS recovery—Time-course experiments demonstrated that the distilled amount of a_{TRIS} during the cold distillation came within one standard deviation of the results of the hot method within 120 min (Fig. 2). In all subsequent experiments we distilled for 120 min to get results that are comparable to the hot method. Fig. 3 shows the results of the hot and cold distillation of the bag incubations of the different sediments. For all sediments tested, the recovery of the cold distillation fell within one standard deviation of the hot method. At a first glance both methods show the same results (Fig. 3). Furthermore, the standard deviation is significantly less when the cold method is applied. This result suggests that the cold method is not only as efficient as the hot method but delivers more precise results.

Blanks—The distillation blank of the cold method ($b_D = B_C + B_D$; 20 ± 2.4 cpm) does not differ significantly from the counter blank

Table 2. Counter blank (b_c), distillation blank (b_D), and sample blank (b_s) obtained with the hot single-step chromium reduction method and the cold distillation method, and calculated minimum detection limit (MDL), where $MDL = \text{Blank} + (3 \times 1\sigma)$

Type of Blank	Blank (cpm)	1 s (cpm)	MDL (cpm)
Hot			
Counter	19	2.6	26.8
Counter + Distillation	34	4.5	47.5
Counter + Distillation + Tracer	104	26	182
Cold			
Counter	19	2.6	26.8
Counter + Distillation	20	2.4	27.2
Counter + Distillation + Tracer	38	1.3	42

($b_c = 19 \pm 2.6$ cpm) (Table 2). The hot method, in contrast, tends to generate a much higher distillation blank ($b_c = 34 \pm 4.5$ cpm) with a larger standard deviation. For the sample blank (b_s), the trend is the same. The cold method has a much lower b_s than the hot method (38 ± 1.3 cpm versus 104 ± 26 cpm).

At any given sulfate reduction rate the amount of a_{TRIS} that is produced per time depends on the specific activity (activity of tracer per mole of sulfate in the sample) of the tracer. An increase in the amount of tracer has its limitations because of the sample blank that is associated with the tracer itself. The distillation of pure $^{35}\text{SO}_4^{2-}$ tracer shows that the blank measurement for both the hot and cold method is proportional to the amount of tracer added (Fig. 4). However, only 0.001‰ of

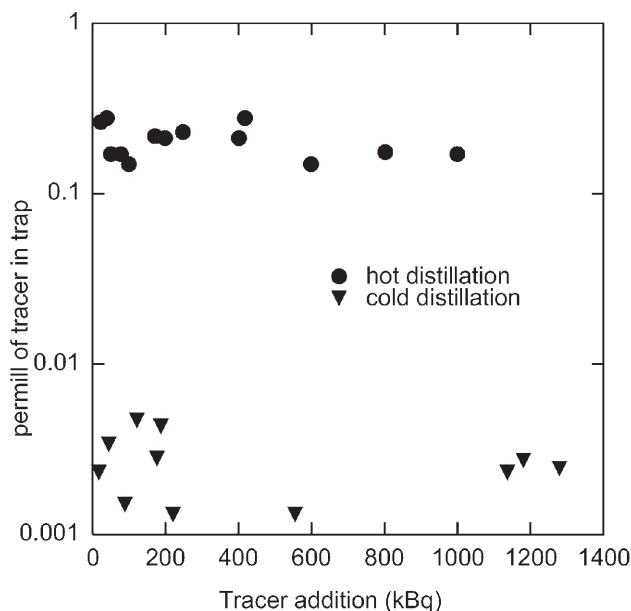


Fig. 4. Relationship between the amount of tracer added and the amount trapped as radiolabeled TRIS in the absence of bacterial sulfate reduction. Hot distillation is shown with circles and cold distillation with triangles.

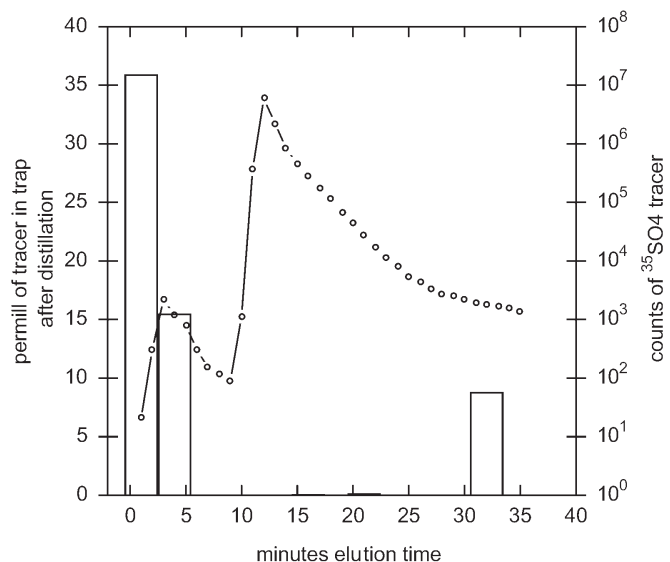


Fig. 5. Separation of the tracer with ion chromatography. The curve shows the distribution of radioactivity versus retention time. The first small peak at 4 min is the contaminating compound and the large one at 12 min is sulfate. The bars show the fraction of the eluted tracer that distilled over in a hot distillation. Note the log scale for radioactivity.

0.01‰ of the tracer is transferred to the ZnAc-trap when nonreduced $^{35}\text{SO}_4^{2-}$ is distilled by the cold method, which is approximately two orders of magnitude lower than the hot distillation. This tracer-associated background is apparently not attributable to sulfate but to some other radioactive sulfur compound. The fact that no radiolabeled TRIS was found in the distillation blank when using the cold method can also be seen as a proof that mixing a sample with 20% ZnAc quickly and efficiently stops all bacterial activity.

Separation of the tracer solution by ion-chromatography provided evidence for the existence of a non-sulfate radioactive compound (curve in Fig. 5). Sulfate separated at about 11 min and 99.95% of the radioactivity was found in the 6- to 24-min fractions. The sample blank (bars in Fig. 5) attributable to the separated sulfate fraction (6 to 24 min) is very low, <0.01‰ of the total counts. A minor peak of radioactivity appeared at 3 min (curve in Fig. 5). Nevertheless, this minor peak of radioactivity contributes most of the sample blank b_s . In the 0- to 6-min fraction about 35‰ of the total counts was measured as sample blank. Thus, 0.05% of the total radioactivity produces a blank that is 3500 times larger than the blank of the remaining 99.95% of the tracer. By counting the fractions separated by IC repeatedly over 2 y, the decay of radioactivity followed the expected decay of ^{35}S (data not shown), proving that the contaminant is a ^{35}S compound. For extremely low SRR we recommend washing the sample twice in 20% ZnAc to remove excess $^{35}\text{SO}_4^{2-}$ before distillation, although preparation time increases accordingly.

Reduction of the distillation and tracer background (B_D and B_T) are the keys to better sensitivity. Therefore, in addition to

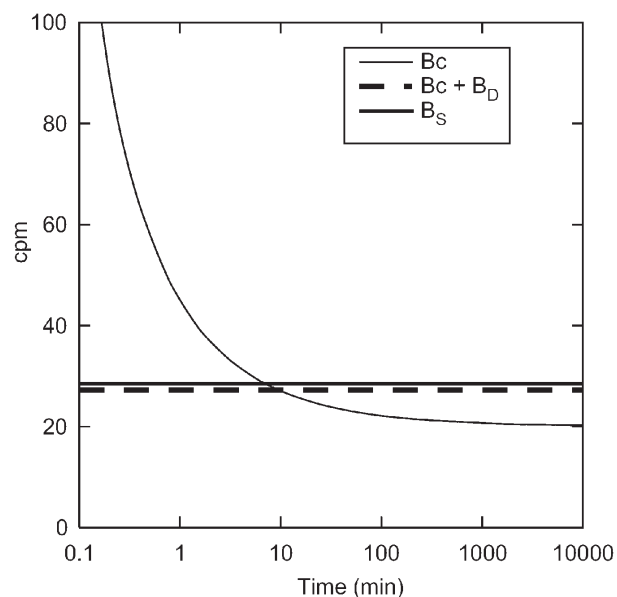


Fig. 6. The influence of counting time on the lower limit of detection of the counter, (LD_{counter}) and on the minimum detection limits (MDL) derived from the distillation blank (b_D) and sample blank (b_S). A counter background count (B_C) of 20 cpm was used for the calculation of LD_{counter} (see text). Distillation blank (b_D) and sample blank (b_S) are 20 ± 2.4 and 38 ± 1.3 cpm, which results in MDLs of 27.2 and 42 cpm, respectively.

lowering the temperature, we sought to improve the distillation setup. To keep the distillation blank to a minimum, all equipment in contact with the sample must be disposable and kept absolutely clean. Cross-contamination was considerably reduced by using only glassware that can either be put into a laboratory dishwasher or is disposable and PEEK tubing that does not absorb any chemicals and can easily be cleaned. Removal of the coolers contributed to a reduction of the distillation blank. The PEEK tubing has a much lower surface area (ca. 1,500 mm²) than the coolers (ca. 95,000 mm²). When comparing the distillation blank from the hot method with those from the cold method (Table 2), the improvement is clear. Not only was b_D (which is equivalent to $B_C + B_D$) reduced but also the standard deviation (from 34 ± 4.5 cpm to 20 ± 2.4 cpm) decreased. The low distillation temperature of the cold method reduces the exposure of metal parts and equipment used in the distillation set-up (e.g., heaters, clamps, and so on) to corrosive acid fumes. A major drawback of the cold method is the use of DMF, which is a toxic organic solvent requiring careful storage and handling. Costs of DMF disposal may also be considerable.

Detection limits—Typically, the minimum detection limit (MDL) is defined as the mean sample blank value, b_S , plus some coverage factor, k , times one standard deviation, σ_{b_S} , of the sample blank signal (Kaiser 1970):

$$\text{MDL} = b_S + (k \times \sigma_{b_S}). \quad (6)$$

The MDL depends on the uncertainty that defines an interval about the measurement result (in this case b_S) within which the value of the measurand (B_S) can be confidently asserted to lie. The measure of uncertainty intended to meet this requirement is termed “expanded uncertainty,” U , and is obtained by multiplying σ_{b_S} by a coverage factor, k (ISO 1995). In general, the value of the coverage factor k is chosen on the basis of the desired level of confidence to be associated with the interval defined by $U = k \times \sigma_{b_S}$. Typically, k is in the range of 2 to 3. When the measurements are normally distributed, and σ_{b_S} is a reliable estimate of the standard deviation of b_S , $U = 2 \times \sigma_{b_S}$ defines an interval having a level of confidence of approximately 95%, and $U = 3 \times \sigma_{b_S}$ defines an interval having a level of confidence greater than 99% (ISO 1995). Both Hurtgen et al. (2000) and Kaiser (1970) observe that blank measurements are not normally distributed, and therefore we may only expect a level of confidence of at least 89% but not more than 95% for $k = 3$. Kaiser (1970) and Skoog and Leary (1992) suggest that k should be set to 3. We thus report a_{TRIS} as:

$$a_{\text{TRIS}} = a_S - b_S \text{ when } a_S \geq [b_S + (3 \times \sigma_{b_S})] \quad (7)$$

or

$$a_{\text{TRIS}} < 3 \times \sigma_{b_S} \text{ when } a_S < [b_S + (3 \times \sigma_{b_S})] \quad (8)$$

where a_S is the activity measured in a sample and a_{TRIS} is the activity of the total reduced inorganic sulfur species (see Eq. 1). The hot method produces a sample blank of 104 ± 26 cpm ($n = 6$), but this value is reduced significantly to 38 ± 1.3 cpm when the cold method is applied (Table 2). The corresponding MDL is 182 cpm for the hot and 41.9 cpm for the cold method. The large standard deviation incurred by the hot distillation method leads to a high MDL. Such high MDLs have previously led to the discouraging situation where sample values sometimes fell well below the MDL even though they may make biogeochemical sense. This problem is well illustrated by the Makran Plateau sediment, which gives counts by the hot method (56 ± 24 cpm g⁻¹) that are significantly below the sample blank (104 ± 26 cpm g⁻¹) and the MDL (182 cpm). The cold method resulted in a similar value, 53 ± 4 cpm g⁻¹, but well above the MDL for the cold method of 41.9 cpm. It should be noted that we have often achieved very low blanks with the hot method. However, it was never a constant or predictable outcome. The advantage of the cold method is that we consistently obtain low and precise blanks.

We also considered whether longer counting times would lower detection limits by decreasing the variability of the counter blank signal (b_C). To assess the effect of counting times and count rates, we use the treatment of Hurtgen et al. (2000) that is based on the seminal work of Currie (1968), where the lower limit of detection, LD (expressed as counts), is defined as

$$\text{LD} = 2.861 + 4.78\sqrt{b_C + 1.36} \quad (9)$$

where $b_c = b_c \times t$, the total counts of the counter blank over the measuring period, and where t = time in minutes at a level of confidence level 95.45% (in this case, radioactivity exhibits a normal distribution, therefore the coverage factor is $k = 2$). The LD_{counter} (cpm) is simply LD/t and represents the signal required in order to state that a signal has been detected with a 4.55% chance of making a Type I error (false positive). Fig. 6 shows the LD_{counter} for background count rates of 20 cpm, which is an upper value for the counter background. It can be seen that longer count times lead to a correspondingly lower LD_{counter} value.

Increasing counting times makes sense only up to a certain point. Our experience is that cpm values of the sample and distillation blanks vary independently from counting time. Thus, after 10 min of counting, these analytical detection limits (MDL as calculated by Eq. 5) exceed the LD_{counter} . Counting times longer than 10 min will not lower the detection limit and the real detection limits that we are likely to be facing are shown as the straight lines in Fig. 6 and that are based on the uncertainty implicit to the distillation itself.

Discussion

We have optimized the distillation procedure for the recovery of reduced sulfur species with particular emphasis on enhancing the sensitivity of sulfate reduction rate measurements with $^{35}\text{SO}_4^{2-}$ radiotracer. The new method combines the advantages of the hot single-step chromium reduction method of Fossing and Jørgensen (1989) with the DMF addition for the passive distillation procedure (Hsieh and Yang 1989) and allows rate measurements over a broad range and down to levels previously not measurable ($<1 \text{ pmol SO}_4^{2-} \text{ cm}^{-3} \text{ d}^{-1}$).

We have reduced cross-contamination and lowered the background considerably to levels that make it possible to detect extremely low rates of sulfate reduction. After the careful consideration of sources of background and imprecision during the hot distillation we observe that the principal gain in sensitivity for the sulfate reduction method derives from the reduction of the distillation temperature. Lower temperatures significantly reduce the background arising from some unknown source within the tracer (B_p). Although clearly a ^{35}S compound, our experiments show that the "contaminant" is definitely not sulfate. However, the background could arise from a Cr-reducible compound that exists in equilibrium with sulfate. A number of higher oxidation state sulfur compounds (e.g., sulfite and thiosulfate) are easily reduced by Cr^{2+} sulfite, for example, is easily reduced to H_2S in acidic chromous chloride solution (Zhabina and Volkov 1978). The sample blank might not only be caused by the contaminant sulfur compound but also through thermochemical sulfate reduction (TSR). Machel (2001) and Trudinger et al. (1985) have shown that TSR can take place in the presence of a strong reducing agent even at temperatures well below 200°C . Divalent chromium is a strong reducer and the walls of the reaction flask certainly reach temperatures above 100°C . When boiling

the sediment slurry in acidic chromous chloride solution, the conditions inside the reaction flask might allow TSR. Although the chromatography experiments (Fig. 5) point to some unidentified compound as the cause of the high B_p , TSR cannot be completely ruled out.

The cold method presented here should be preferred when low counts (i.e., close to the sample blank) are expected. The cold method has some drawbacks, mainly the longer distillation time and the use of DMF, which is toxic and needs special disposal. The turnover of samples is somewhat more time-consuming, and this factor should be taken into consideration when large numbers have to be processed. If the number of counts is expected to be high and the minimum detection limit not a problem, the hot method may have the advantage of a higher sample turnover with less toxic material (no DMF). Nevertheless, the cold method has wider applications than just measuring SRR on deeply buried and low-activity sediments. Higher sensitivity also allows for the reduction of incubation times, thereby minimizing the artifacts caused by changes in the sample during incubation (e.g., sulfide oxidation).

However, an increase in sensitivity cannot be achieved by the new distillation method alone but also through a vigorous evaluation of the counting conditions and the statistical treatment of the data. The different sources of the blank have to be quantified for the individual distillation and counting set-up in order to be able to work on a sound statistical basis for detection of SRR on the limit of detection.

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