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Measurement of bacterial sulfate reduction in sediments: Evaluation of a single-step chromium reduction method

HENRIK FOSSING & BO BARKER JØRGENSEN
Institute of Ecology and Genetics, University of Aarhus Ny Munkegade, DK-8000 Aarhus C, Denmark

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Abstract. A procedure which includes the Total Reduced Inorganic Sulfur (TRIS) in a single distillation step is described for the radiotracer measurement of sulfate reduction in sediments. The TRIS includes both Acid Volatile Sulfide (AVS: H_2S + FeS) and the remaining Chromium Reducible Sulfur (CRS: S^0, FeS_2). The single-step distillation was simpler and faster than the consecutive distillations of AVS and CRS. It also resulted in higher (4–50%) sulfate reduction rates than those obtained from the sum of ^{35}S in AVS and CRS. The difference was largest when the sediment had been dried after AVS but before CRS distillation. Relative to the ^{35}S-AVS distillation alone, the ^{35}S-TRIS single-step distallation yielded 8–87% higher reduction rates. The separation and recovery of FeS, S^0 and FeS_2 was studied under three distillation conditions: 1) cold acid, 2) cold acid with Cr^{2+}, and 3) hot acid with Cr^{2+}. The FeS was recovered by cold acid alone while pyrite was recovered by cold acid with Cr^{2+}. A smaller S^0 fraction, presumably of the finer crystal sizes, was recovered also in the cold acid with Cr^{2+} while most of the S^0 required hot acid with Cr^{2+} for reduction to H_2S.

Introduction

Direct radiotracer measurements of sulfate reduction rates were first done by Sorokin (1962), who mixed $^{35}SO_4^{2-}$ into homogenized sediment, and by Ivanov (1968), who injected the radiotracer into subsamples of sediment. Multiple $^{35}SO_4^{2-}$ injections into intact cores were later used in order to retain the undisturbed stratification of the sediment (Jørgensen 1977; 1978). All these techniques were based on the assumption that $H_2{}^{35}S$ produced from $^{35}SO_4^{2-}$ was retained as Acid Volatile Sulfide (AVS), i.e., free sulfide and FeS, which could be distilled off when the sediment was acidified with HCl.

Howarth and coworkers demonstrated that the reduced ^{35}S was also incorporated into the non-acid volatile sulfur compounds, especially pyrite (Howarth 1979; Howarth & Teal 1979; Howarth & Giblin 1983). In salt marsh sediments they recovered 70–95% of the reduced ^{35}S in pyrite and elemental sulfur and concluded that the sulfate reduction measurements based only on AVS radioactivity would underestimate the true reduction rates accordingly.

In the early measurements of Howarth and coworkers, radioactive pyrite was oxidized to sulfate by aqua regia after acid volatile sulfide had been stripped from the sediment. Other authors found the possibility of contamination from non-reduced sulfate to be a potential problem of this technique (Westrich 1983; King 1983). A more selective method was the reduction of non-acid volatile sulfur by Cr^{2+} in acid solution (Zhabina & Volkov 1978). This Chromium Reducible Sulfur (CRS) comprised both elemental sulfur and pyrite. Studies of sulfate reduction rates in a range of marine sediments and salt marshes have shown that 10–50% or even more of the reduced radiolabel is recovered from CRS (Lein et al. 1982; Westrich 1983; Howarth & Jørgensen 1984; Howes et al. 1984; King et al. 1985). The percentage varies widely depending on the type of sediment and on the sulfur chemistry (Thode-Andersen and Jørgensen 1989).

The mechanism of ^{35}S incorporation into elemental sulfur and pyrite during short-term incubations is not yet clear, but ^{35}S labelled CRS obviously has to be taken into account in measurements of sulfate reduction rates. In earlier measurements, a two-step procedure was used which separately determined the radioactivities of AVS and CRS. In most sulfate reduction studies, however, a faster single-step procedure, which includes all reduced inorganic sulfur, would be preferable. We describe here such a single-step chromium reduction method and evaluate it relative to earlier techniques.

Materials and methods

Sediment cores were sampled near Aarhus, Denmark, from four shallow water locations which ranged from marine to fresh water: Aarhus Bay, Kalø Lagoon, Kysing Fjord, and Odder Stream.

Measurements of sulfate reduction

A two-step and a single-step method for the measurement of sulfate reduction were compared. By the two-step method, acid volatile sulfide and chromium reducible sulfur were sequentially distilled from the sediment. The fraction of $^{35}SO_4^{2-}$ reduced during incubation was calculated from the sum of ^{35}S in AVS and CRS. By the single-step method the total reduced inorganic sulfur was distilled simultaneously with Cr^{2+} and the $^{35}SO_4^{2-}$ reduced was calculated from the total $H_2{}^{35}S$ released.

Sulfate reduction was measured in undisturbed sediment cores of 3 cm diameter by the core injection technique (Jørgensen & Fenchel 1974; Jørgensen 1978). A volume of 2 μl carrier-free $^{35}SO_4^{2-}$ (70 kBq) was injected at specific depths into five intact cores from each station. The sediment was incubated for 18–24 h at room temperature before it was cut into segments and transferred to 20 ml of 20% (w/v) zinc acetate (ZnAc) and frozen to terminate incubation and fix sulfides. The reduced sulfur was then distilled as H_2S from the sediment into ZnAc traps as described below and the radioactivities of SO_4^{2-} and of precipitated ZnS were determined. Sulfate concentrations in porewater were determined gravimetrically in 3–4 parallel cores from each station after precipitation with Ba^{2+} (American Public Health Association, 1971).

The Sulfate Reduction Rates (SRR) were calculated according to the equation:

$$\mathrm{SRR} = \frac{(\mathrm{SO_4^{2-}})a \cdot 24 \cdot 1.06}{(A + a)h} \ \mathrm{nmol\ SO_4^{2-}\ cm^{-3}\,d^{-1}}$$

where a is the total radioactivity of ZnS, A is the total radioactivity of SO_4^{2-} after incubation, h is the incubation time in hours, (SO_4^{2-}) is the sulfate concentration in nmol per cm^3 sediment, and 1.06 is a correction factor for the expected isotope fractionation (Jørgensen & Fenchel 1974).

Only sediment from one or two depth intervals were used for each station. Segments from each depth interval were pooled from the five cores and homogenized.

Two-step method

Acid volatile sulfide was distilled from a weighed amount of homogenized sediment (Jørgensen & Fenchel 1974; Jørgensen 1978). The sediment was acidified with 1 N HCl. AVS was liberated as H_2S and stripped from the slurry for 30 min using N_2 as a carrier gas. The H_2S was precipitated in two sequential traps containing 10 ml 5% ZnAc, buffered with 0.1% acetic acid, and with a drop of antifoam. More than 98% of the distilled H_2S was recovered as ZnS in the first trap. After distillation the two traps were pooled, 5 ml was subsampled, mixed with 5 ml of scintillation fluid (Dynagel, Baker Chemicals), and ^{35}S was counted. The radioactivity of the sulfate remaining in the reaction vessel was similarly determined on a 5 ml subsample. The concentration of AVS was determined spectrophotometrically (Cline 1969) on subsamples of ZnS from the ZnAc traps.

Chromium reducible sulfur was determined in the sediment slurry remaining from AVS distillation. The slurry was washed twice with seawater and centrifuged to remove $^{35}SO_4^{2-}$. The mud was dried at 90 °C overnight and ground in a mortar. A 0.5–1 g subsample was mixed with 5 ml ethanol in a 100 ml reaction flask (described below). After the reaction flask had been degassed for 20 min with N_2, 16 ml of 1 M Cr^{2+} in 0.5 N HCl and 8 ml of 12 N HCl were added (Westrich 1983; Howarth & Jørgensen 1984; Canfield et al. 1986). The distillation of CRS into ZnAc traps proceeded for 15 min at room temperature followed by 45 min of gently boiling. The concentration and radioactivity of CRS were determined as for AVS.

Single-step method

The homogenized sediment was centrifuged and $^{35}SO_4^{2-}$ radioactivity was measured in a subsample of the supernatant. The sediment pellet was washed twice with seawater to remove $^{35}SO_4^{2-}$. The washed sediment was homogenized and 1–2 g transferred to a reaction flask and mixed with 5 ml distilled water and 5 ml ethanol. The reaction flask was supplied with a gas-bubbling tube, a condenser, and two ZnAc traps (cf. Canfield et al. 1986). The reaction flask was degassed for 20 min with N_2, 16 ml of 1 M Cr^{2+} in 0.5 N HCl and 8 ml of 12 N HCl were added, and the sediment slurry was gently boiled for 40 min. During this distillation the Total Reduced Inorganic Sulfur (TRIS) was dissolved and carried as H_2S to the ZnAc traps. Concentration and radioactivity were determined as before.

Chromium reduction procedure

The highly reactive Cr^{2+} solution was produced from the more stable Cr^{3+} by percolating 1 M $CrCl_3,6H_2O$ in 0.5 N HCl through a Jones reductor (Peters et al. 1974) with amalgamated zinc granules:

$$2\,Cr^{3+} + Zn \longrightarrow 2\,Cr^{2+} + Zn^{2+}$$

An efficient reduction was verified by a color change from dark green (Cr^{3+}) to bright blue (Cr^{2+}). The Jones reductor was built from a glass-column (40-cm long, 1.5 cm ID) with an integral sinter at the bottom and stopcocks in both ends. The granular zinc (0.3–1.5 mm grain size) was washed three times with 1 N HCl and twice with dem. H_2O. The zinc was amalgamated for a few minutes with a saturated solution of $HgCl_2$ (about 0.25 M), transferred to the glass column, and washed with three column volumes of 0.5 N HCl. The reduced chromium solution was sucked into large polypropylene syringes in which it stayed reduced (bright blue) for several weeks.

An alternative and faster method of producing larger volumes of Cr^{2+} solution is simply to fill a glass bottle with 1 N HCl-rinsed “mossy zinc” (Aldrich Chemicals) and then fill the bottle with the Cr^{3+} solution (1 M

$CrCl_3,6H_2O$ in 0.5 N HCl) under continuous flow of N_2. The mossy zinc does not need to be amalgamated. The chromium is reduced to Cr^{2+} within 10 min and is kept under N_2 until it is drawn into syringes through an outlet at the bottom of the bottle. After use, the mossy zinc can be regenerated by washing with 1 N HCl.

Preparation of FeS, FeS_2, and S^0

The three sulfur minerals were prepared for controls on the selectivity of different distillation procedures.

Ferrous monosulfide, FeS, was prepared according to Brock and O'Dea (1977) by mixing solutions of ferrous iron (16.7 g $FeSO_4,7H_2O$/100 ml) and sulfide (14.4 g $Na_2S,9H_2O$/100 ml). The precipitated FeS was washed 5 times with boiled, distilled water to remove free sulfide.

Pyrite, FeS_2, was prepared by adding 2–3 g "flowers of sulfur" to a 100 ml suspension of 2–3 g freshly precipitated FeS (not washed) in a 120 ml serum vial. The flask was bubbled with N_2, sealed, and incubated at 65 °C for 4 days and at 85 °C for another 4 days (Berner 1969, 1970; Sweeney & Kaplan 1973). The FeS_2 was washed three times with 1 N HCl to remove free sulfide and FeS and then three times with distilled water. The FeS_2 was dried at 90 °C overnight and extracted several times with CS_2 until all residual S^0 had been removed. X-ray crystallography of the dried precipitate showed that FeS_2 was the only detectable mineral.

Elemental sulfur, S^0, was prepared in three different ways. A single sulfur gradule of a few mm^3 volume was prepared directly in the reaction flask by evaporation of 10 ml CS_2 containing 10 mg of dissolved flowers of sulfur. Colloidal sulfur was obtained by boiling excess flowers of sulfur (1 g) in 50 ml acetone for three minutes. The supernatant acetone was mixed with distilled water, 1:3, whereby a fine suspension of S^0 was obtained with a particle size of 0.2–0.5 μm. Finally, a saturated solution of S^0 in acetone was used directly.

Control experiments

Controls were made of the different distillation methods, of their time course and selectivity, as well as of the contamination level from $^{35}SO_4^{2-}$ and of the effect of ethanol. Six control experiments are described here.

1. The time course of distillation was studied in the homogenized sediment from brackish water localities, Kalø Lagoon (0–3 cm depth) and Kysing Fjord (5–8 cm depth). The distillation proceeded in three steps, each of 40 min duration, and the sulfide traps were changed every 5 min. A) Distilla-

tion with cold acid: About 2 cm^3 of homogenized sediment was mixed with distilled water, flushed with N_2, and 12 N HCl was added to a final concentration of 2 N. B) Distillation with cold acid and Cr^{2+}: After the first step, 16 ml of 1 M Cr^{2+} in 0.5 N HCl and 8 ml of 12 N HCl were added under continued N_2 flow. C) Distillation with hot acid and Cr^{2+}: The reaction flasks were heated and distillation continued under gentle boiling. For each set of ZnAc traps, both in this and in the following control experiments, the amount and radioactivity of sulfide were determined.

2. A time course of distillation was done on homogenized sediment from Aarhus Bay (6–8 cm depth) and Kalø Lagoon (0–3 cm depth) according to the described single-step method with hot acid and chromium. Sulfide traps were again changed every 5 min and distillation continued for 60 min.

3. The three-step time course of experiment 1 was repeated with 100–500 μmol of each of the three prepared sulfur minerals, FeS, FeS_2, and S^0. Sulfide traps were changed only once after each step.

4. The three-step time course experiment was done also with sediment with and without extraction of elemental sulfur prior to distillation. The purpose was to demonstrate at which step the elemental sulfur was released during the distillations. Elemental sulfur was extracted with CS_2 from homogenized sediment from Kalø Lagoon (0–3 cm depth) and Kysing Fjord (5–8 cm depth) as described by Troelsen and Jørgensen (1982). The S^0 radioactivity in the CS_2 extract was determined. Sulfide traps were changed after each step.

5. The possible contamination from unreacted $^{35}SO_4^{2-}$ in the sulfide traps was checked with homogenized sediment from Aarhus Bay (2–4 cm depth) and Odder Stream (4–6 cm depth). Additional $^{35}SO_4^{2-}$ was added to 2 g of sediment to a final radioactivity of 800 kBq. Prior to distillation, the sediment was washed several times with distilled water. The single-step method was used in the distillations and the carry-over of $^{35}SO_4^{2-}$ via aerosols into the sulfide traps or the possible reduction by chromium were determined.

6. The effect of drying the sediment and of ethanol addition on the distillation efficiency was studied in sediments from Kysing Fjord (5–8 cm depth) and Kalø Lagoon (0–3 cm depth). Acid volatile sulfide was first distilled from both sediments. The sediments were then washed twice, and half of each was dried at 90°C overnight and ground in a mortar. The other half remained wet. Chromium reducible sulfur was distilled from the dry and the wet sediment with either 5 ml of ethanol or 5 ml of water added to the reaction flask. The total sulfide radioactivity was determined in the ZnAc traps from each of the four distillations.

Results

The sediment types studied ranged from silt to sand and the salinities from marine to fresh water:

— Aarhus Bay: brown to gray silt, brackish-marine (23‰).

— Kalø Lagoon: black sand and decomposing eelgrass at the surface and black sand below, brackish-marine (23‰).

— Kysing Fjord: brown to black sand, brackish (12–20‰).

— Odder stream: brown silt, fresh.

Table 1 shows the concentrations of sulfate and of the main reduced, inorganic sulfur pools in the sediments which were used for experiments. The Kalø Lagoon sediment was covered by a microbial film of cyanobacteria as well as purple and colorless sulfur bacteria, typical of a sulfuretum. A high concentration of elemental sulfur, > 15 μmol cm^{-3}, was measured at the surface of this sediment by Troelsen & Jørgensen (1982). At the other stations, elemental sulfur concentrations were small relative to pyrite and constituted only < 5% of the CRS.

The sulfate reduction rates in the same sediments as determined by the single-step method (SRR_{TRIS}) are presented in Table 2. For comparison, the rates calculated from the two-step method ($SRR_{AVS+CRS}$) or from AVS distillation alone (SRR_{AVS}) are expressed as percent of the SRR_{TRIS}.

Sulfate reduction rates measured at all four stations were in the high range for coastal sediments (cf. Skyring 1987). Especially the organic-rich sediment of Kalø Lagoon had very intensive sulfur cycling. When sulfate reduction was calculated from AVS radioactivity alone, the rates were only 53–93% of those obtained from TRIS distillation. Even when CRS radioactivities were added, as in the two-step method, the rates were lower than

Table 1. Concentrations of sulfate, acid volatile sulfur (AVS), chromium reducible sulfur (CRS, measured on dried sediment), and total reduced inorganic sulfur (TRIS) as determined in the sediment types studied. Concentrations are the mean of 4 measurements ± s.d. of the mean.

Location	Depth cm	SO_4^{2-}	AVS	CRS	TRIS
		μmol S-eqv. cm^{-3}			
Aarhus Bay	2–4	14.8 ± 1.0	19.3 ± 1.3	50.5 ± 2.9	73.9 ± 1.7
Aarhus Bay	6–8	13.9 ± 0.1	17.9 ± 0.5	151.8 ± 14.7	175.0 ± 7.7
Kalø Lagoon	0–3	12.2 ± 1.2	15.8 ± 0.8	12.4 ± 0.5	33.2 ± 1.0
Kalø Lagoon	6–8	5.7 ± 0.8	6.2 ± 0.4	15.4 ± 0.9	35.3 ± 2.3
Kysing Fjord	5–8	7.8 ± 0.2	11.0 ± 0.4	65.8 ± 5.4	93.1 ± 1.8
Odder Stream	4–6	0.09 ± 0.03	19.1 ± 0.6	13.3 ± 0.5	35.0 ± 1.7

Table 2. Sulfate reduction rates in the sediment types studied as determined by the single step method (SRR_{TRIS}). Rates calculated from the two-step method ($SRR_{AVS+CRS}$) and from AVS distillation (SRR_{AVS}) are expressed as percent of SRR_{TRIS}. Rates are the mean of 4 measurements ± s.d. of the mean. By Student's t-test it was tested whether SRR_{AVS} and $SRR_{AVS+CRS}$ differed significantly from SRR_{TRIS} at the 5% (+), 1% (+ +), or 0.1% (+ + +) level or did not differ significantly (−).

Location	Depth cm	SRR_{TRIS} nmol cm^{-3} d^{-1}	$SRR_{AVS+CRS}$	SRR_{AVS}
			% of SRR_{TRIS}	
Aarhus Bay	2–4	74.3 ± 0.6	87.3 ± 3.8 (+ + +)	69.9 ± 3.4 (+ + +)
Aarhus Bay	6–8	33.5 ± 0.7	89.9 ± 2.7 (+ + +)	69.8 ± 2.1 (+ + +)
Kalø Lagoon	0–3	833.6 ± 17.3	89.6 ± 4.2 (+ +)	84.0 ± 4.1 (+ + +)
Kalø Lagoon	6–8	206.3 ± 0.8	94.4 ± 5.3 (−)	86.6 ± 5.2 (+ +)
Kysing Fjord	5–8	84.0 ± 2.4	67.1 ± 2.8 (+ + +)	53.5 ± 0.9 (+ + +)
Odder Stream	4–6	60.6 ± 1.1	96.3 ± 5.2 (−)	92.6 ± 5.5 (+)

those of the single-step method. In two sediments, however, the difference was not statistically significant.

Control experiments

1. The time course of distillation was done in three steps on sediments from Kalø Lagoon (Fig. 1, I) and Kysing Fjord (Fig. 1, II). The figures show the sequence at which the reduced sulfur pools were released as H_2S and trapped in ZnAc. Distillation was completed (> 95%) within 20–30 min at each step. Both the radioactivity and the total pool size of sulfur is shown for the distillations. In sediment from Kalø Lagoon, the largest sulfur pools were obtained by distillation in cold acid without and with Cr^{2+}. At Kysing Fjord most of the sulfur was obtained by cold distillation with Cr^{2+}. The AVS fraction had the highest total radioactivity of the three pools at both stations.

2. The time course of a single-step distillation is shown for Kalø Lagoon sediment in Fig. 2. Within 20 min, > 95% of the total released H_2S had been distilled. After 40 min distillation, < 1% additional H_2S was released over the remaining period. Similar results were obtained for Aarhus Bay sediments (data not shown).

3. A three-step distillation of the pure sulfur minerals demonstrated the selectivity of each step (Table 3). About 98% of the FeS was distilled during the first step with cold HCl. The remaining 2% required Cr^{2+} addition for distillation. This small fraction expectedly represented elemental sulfur and pyrite, which were present as contaminants in the FeS, or which were produced during the distillation.

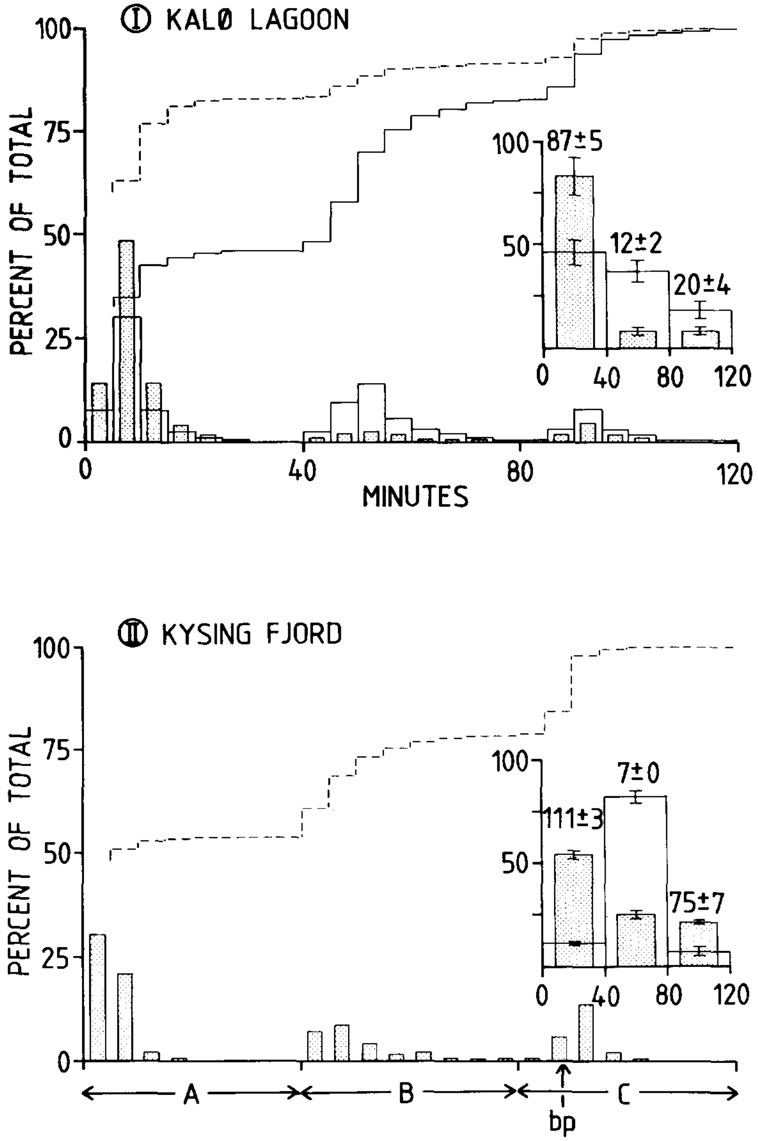

Fig. 1. Time course of distillation done in three steps on sediment from I: Kalø Lagoon and II: Kysing Fjord. Distillation step A) 0–40 min: cold HCl; B) 40–80 min: cold HCl with Cr^{2+}; C) 80–120 min: hot HCl with Cr^{2+}. (bp: boiling point). Shaded columns: Radioactivity. Unshaded columns: Pool size of sulfur. Broken line: Radioactivity (cumulative). Solid line: Pool size of sulfur (cumulative). Data are the mean of three distillations. Insert: Sum of radioactivity and pool size, respectively, calculated for each distillation step. Numbers show specific radioactivities (cpm/μmol S-equivalent; mean ± s.d.; n = 3).

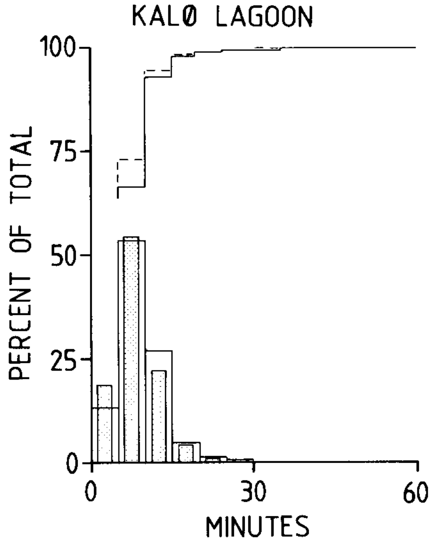

Fig. 2. Time course of distillation done in a single step (TRIS) on sediment from Kalø Lagoon. Shaded columns: Radioactivity. Unshaded columns: Pool size of sulfur. Broken line: Radioactivity (cumulative). Solid line: Pool size of sulfur (cumulative). Data are the mean of three distillations.

The pyrite was quantitatively recovered during the cold distillation with Cr^{2+}. Thus, at least the pyrite produced under laboratory conditions did not require heating to become reduced to H_2S.

The recovery of elemental sulfur was strongly dependent on its form. The larger granules of sulfur were recovered only inefficiently and only in hot HCl and Cr^{2+}. Even after 4 h of distillation, < 10% has been reduced. Also the reduction of colloidal sulfur required heating, but the recovery was much

Table 3. Selectivity of the different distillation procedures on three sulfur minerals: ferrous monosulfide, pyrite, and elemental sulfur. The three distillation steps are: A) cold HCl; B) cold HCl with Cr^{2+}; and C) hot HCl with Cr^{2+}. Percent recovery are the mean of 3 measurements ± s.d. of the mean.

Sulfur Mineral	Cold HCl	Cold HCl + Cr^{2+}	Hot HCl + Cr^{2+}	Total
	% Recovery			
FeS	97.3 ± 0.3	0.9 ± 0.0	1.8 ± 0.0	100.0 ± 0.3
FeS_2	0.0 ± 0.0	100.0 ± 8.4	0.0 ± 0.0	100.0 ± 8.4
S^0, Granule	0.0 ± 0.0	0.0 ± 0.0	7.6 ± 3.1	7.6 ± 3.1
S^0, Colloidal	0.0 ± 0.0	2.0 ± 0.0	44.3 ± 2.8	46.3 ± 2.8
S^0, Dissolved	0.0 ± 0.0	91.4 ± 2.8	4.0 ± 0.0	95.4 ± 2.8

higher, 46%. Only when the elemental sulfur was dissolved in acetone was it reduced in cold HCl and Cr^{2+}. The total recovery was then 95%. Thus, the particulate form of elemental sulfur, as is present in sediments, will expectedly require heating for distillation.

4. This observed selectivity of the three steps of distillation was verified in sediments from Kalø Lagoon and Kysing Fjord (Fig. 3, I + II). Distillation with and without prior extraction of the elemental sulfur with CS_2 primarily showed a difference in the hot chromium fraction (step C). The chromium reducible sulfur, which remained in this fraction after prior S^0 extraction, was equal to the amount of S^0 remaining in non-removed CS_2. In Kalø Lagoon sediment, the decrease in the hot chromium reducible sulfur was just equal to the amount of S^0 extracted. In the Kysing Fjord sediment, the large decrease in the hot chromium fraction plus the small decrease in the cold chromium together equalled the S^0 extracted. Thus, all the sulfur reduced during step C could be accounted for as S^0. Consequently, step B included the pyrite fraction as well as a minor part of the elemental sulfur. The AVS fraction (step A) was not significantly affected by the S^0 extraction.

5. The carry-over of label from radioactive sulfate during single-step distillations was $0.030 \pm 0.004\%$ ($n = 4$) as calculated relative to labelled

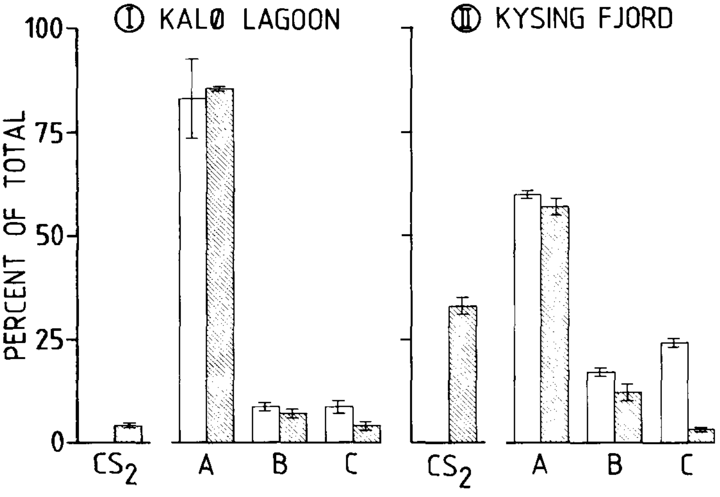

Fig. 3. Distillation with or without prior CS_2-extraction of elemental sulfur. A three step distillation was done on sediment from I: Kalø Lagoon and II: Kysing Fjord. CS_2: Radioactivity of CS_2-extracted S^0. Distillation step A) cold HCl; B) cold HCl with Cr^{2+}; and C) hot HCl with Cr^{2+}. Hatched columns: CS_2-extracted sediment, radioactivity. Unhatched columns: Non-CS_2-extracted control, radioactivity. Data are the mean of three distillations; bars show ± s.d. of mean.

sulfate added to sediment slurries. This contamination would give an erroneous increase in the calculated sulfate reduction rate of up to 6 nmol cm^{-3} d^{-1} in the sediments studied here. Such a background can create a serious error, especially in offshore sediments with a low bacterial activity. By washing the sediment 2 or 4 times prior to distillation, the concentration of radioactive sulfate was reduced by 1000-fold and 10,000-fold, respectively, and the carry-over was thus $< 3 \times 10^{-7}$ and $< 3 \times 10^{-8}$ of the initial sulfate radioactivity.

6. Drying of the sediment after AVS distillation but before chromium distillation showed the following effects. During the cold chromium distillation with ethanol (step B), which primarily included the pyrite fraction, there was no effect of drying. The recovery from dried sediment was 99.5 ± 5.9% of that from wet sediment (mean of 3 measurements ± s.d. of the man). During the hot chromium distillation with ethanol (step C), however, only 30.4 ± 2.1% was recovered from the dried sediment relative to the wet sediment. Since this fraction included mostly elemental sulfur, the S^0 recovery was adversely affected by drying of the sediment, even in the presence of ethanol.

The addition of ethanol facilitates the reflux condensation during distillation but had no effect on the combined recovery of H_2S from step B and C in wet sediment. The recovery with ethanol added was 100.0 ± 1.6% of that without. When the sediment had been dried, however, the recovery was only 63.7 ± 2.6% and 55.2 ± 1.6%, respectively, depending on whether ethanol was added or not.

As a consequence of these control experiments, the single-step method was designed without drying of the sediment and with the addition of ethanol.

Discussion

The published procedures of sulfate reduction measurements, in which the formation of radio-labelled pyrite has been taken into account, have all involved the separate determination of acid volatile sulfide, pyrite, and sometimes also elemental sulfur. The main purpose of these separations has been to demonstrate the formation of labelled non-acid volatile sulfide during short-term incubations.

Methodological studies have revealed several systematical errors inherent in earlier distillaion techniques. The analysis of acid volatile sulfide tends to underestimate its pool size and radioactivity due to sulfide oxidation by Fe^{3+} released by the added acid (Berner 1964, 1974). Some of the sulfide may be oxidized to S^0, which would be recovered by the subsequent CRS distillation, while some may be oxidized completely to SO_4^{2-}. To avoid this artifact, distillations have been done with Sn^{2+} or Ti^{3+} added to the sediment slurry (Chanton and Martens, 1985; Albert 1985). These metals reduce Fe^{3+} to Fe^{2+} and were found to increase sulfide recovery by 20% and 38%, respectively, in the sediments tested. Also heating of the sediment slurry increases the recovery. A problem of metal addition combined with heating is, however, that sulfur species in addition to AVS, e.g. S^0, become partly reduced and volatilized. Our earlier control experiments have shown that the AVS reoxidation to S^0 is small, only 1–5% of reduced ^{35}S, in the present separations (Howarth & Jørgensen 1984; Thode-Andersen & Jørgensen 1989).

The formation of radioactive pyrite and elemental sulfur during $^{35}SO_4^{2-}$ reduction has now repeatedly been documented, although the mechanism of tracer incorporation into these compounds is still not clear. It can represent true in situ formation of the two minerals but it can also be affected by isotopic exchange reactions of ^{35}S between the reduced sulfur pools (Fossing and Jørgensen, in prep.). The specific radioactivities calculated for the sulfur pools extracted by the three distillation steps is shown in Fig. 1 (inserts). The H_2S is produced from bacterial sulfate reduction during incubation and has the same average specific radioactivity as the sulfate pool, i.e., 52.000 and 81.000 cpm/μmol S-equivalent, respectively, for Kalø Lagoon and Kysing Fjord. The specific radioactivities in Fig. 1 demonstrate how radiotracer is distributed into the three pools from the initially produced $H_2{}^{35}S$. The AVS has the highest specific radioactivity as this pool includes both the produced $H_2{}^{35}S$ and rapidly precipitated $Fe^{35}S$. From AVS the ^{35}S is transformed into FeS_2 and S^0 during incubation and the radiotracer is thereby further diluted (the specific radioactivity is decreased) in accordance with the pool sizes.

Since in many studies it is the total rate of sulfate reduction, which is of interest, a single-step distillation procedure as described here provides a simpler and also more accurate method. Both the oxidation artifact during AVS distillation and the loss due to drying are avoided. A two-step procedure in which AVS and CRS were determined separately thus yielded only 67–96% of the sulfate reduction rate measured by the single-step procedure (Table 2). The difference was mostly due to inefficient S^0 reduction after drying of the sediment and to a lesser extent due to losses during AVS distillation. Determination of the AVS pool alone yielded 54–93% of the single-step procedure.

Most radiotracer studies of sulfate reduction have applied an active distillation procedure similar to here in which the H_2S evolved upon acidification was stripped into traps by a stream of inert gas. A passive distillation during which the H_2S diffuses into the trap within a closed reaction vessel is a possible alternative (e.g., Howarth & Giblin 1983; Rosser and Hamilton 1983; Albert 1985). We are, however, not aware of studies which have reported on passive distillation in connection with chromium reduction. Such a technique would allow a larger number of samples to be processed simultaneously. By our single-step active distillation (20 min preflushing with N_2 and 40 min distillation) in five parallel chambers we can currently process 30–35 samples per day.

Several control experiments are important before the routine application of this technique. The efficiency and time course of H_2S distillation must be checked to assure complete recovery of chromium reducible sulfur. Active distillation times ranging from 22 min (Cutter & Oatts 1987) to 2 hours (Howes et al. 1984; Canfield et al. 1986) have been reported. Also a time course experiment of sulfate reduction rates should be carried out to check the linearity of the process and the zero time contamination of radioactive sulfate in the reduced sulfur pool. The addition of non-radioactive carrier sulfide in the form of Na_2S or ZnS can also be recommended when only the H_2S radioactivity and not its concentration is being measured, especially for sediments with a low AVS content.

Separation of sulfur pools

Four groups of sulfur compounds seem to be of significance in relation to radiotracer measurements of sulfate reduction: free sulfide, iron monosulfides, pyrite, and elemental sulfur. In the present study we separated three sulfur pools by consecutive distillations in, A) cold acid, B) cold acid with Cr^{2+}, and C) hot acid with Cr^{2+}.

The "cold acid" step includes free sulfide and iron monosulfides with the probable exception of well crystallized forms such as greigite (Table 3). This pool had the highest specific radioactivity and also carried most of the reduced ^{35}S but was generally not the largest sulfur pool (Fig. 1).

The "cold acid with Cr^{2+}" step included pyrite and a varying amount of elemental sulfur. According to Berner (1969, 1970), pyrite prepared by the present technique is mostly framboidal similar to that found in natural sediments. This was confirmed by microscopic examination. The laboratory-produced pyrite was recovered with cold acid with Cr^{2+} while the recovery of elemental sulfur decreased with increasing crystal size (Table 3). Also in sediments, the pyrite was recovered mostly at this step (Fig. 3). Similar observations were done by Cutter and Oatts (1987) in marine sediments. We thus conclude that heating, such as described in the procedures of Zhabina & Volkov (1978), is not necessary for the chromium reduction of pyrite.

The higher reactivity of pyrite than of elemental sulfur towards Cr^{2+} reduction may be explained by molecular-orbital theory (Luther 1987). The Cr^{2+} cannot attack S^0 efficiently because of the S_8 ring which has every sulfur atom bound to two other sulfur atoms. The S_8 is an electrophile and Cr^{2+} is a cation and chemical attack (bonding) is thus likely to be minimal between them. Reduction should be through an outer sphere mechanism. This is not the case for pyrite in which one sulfur is always exposed at the surface and is a nucleophile. Thus, this sulfur atom can bind Cr^{2+}. Close approach of the reactants is likely and electron transfer should be inner sphere. The electron transfer is faster because only one S–S bond must be broken per sulfur atom in pyrite rather than two bonds per sulfur atom in S_8. For S_8, heating would therefore be necessary to lower the activation energy for reaction with Cr^{2+}.

The "hot acid with Cr^{2+}" step included that part of the elemental sulfur which had not already been reduced. Extraction of elemental sulfur by CS_2 removed most of this sulfur in the S^0-rich sediment of Kysing Fjord (Fig. 3, II). Canfield et al. (1986) found 92% recovery of pure S^0 by hot Cr^{2+}, but did not specify the chemical form of the S^0. The form of elemental sulfur present in marine sediments is poorly known and is probably quite heterogeneous with respect to crystal size, surface properties, chemical composition, etc. The dependence of the recovery on the degree of heating can therefore be expected to be similarly variable. In order to determine the formation of radio-labelled elemental sulfur it is therefore necessary to extract this pool with organic solvents before the distillations. Several solvents such as hexane, CS_2, CCl_4, acetone, and ethanol have been used. CS_2 is an excellent solvent but cannot be used if pool sizes are determined during subsequent hot Cr^{2+} reduction. During heating a small fraction of the CS_2 decomposes to elemental carbon and elemental sulfur and the latter is then reduced by the Cr^{2+}.

Many earlier sulfur distillations with chromium have been done on dried sediments. Canfield et al. (1986) compared chromium distillations of fresh and dried marine sediment and found no significant difference in the sulfur pools. We found that only 30% of the radioactive elemental sulfur was recovered by hot chromium reduction after the sediment had been dried, whereas the pyrite recovery was not affected by drying. The effect will depend on the S^0 concentration and will be less evident when the large total sulfur pool is analyzed, but it is important if the incorporation of radio-label into specific sulfur pools is studied.

Some of the radiolabelled $H_2{}^{35}S$ produced during incubation may also have been incorporated into sulfur pools other than AVS, S^0, and FeS_2, but such pools have not been separately determined. Polysulfide is commonly found in the porewaters of marine sediments (Boulegue et al. 1982). It is formed in the presence of H_2S and S^0 and plays an important role for the isotopic exchange between these two compounds (Voge 1939; Fossing & Jørgensen, in prep.). The polysulfide decomposes to H_2S and S^0 upon acidification and will thus be recovered during the single-step distillation. Thiosulfate is an important product of sulfide oxidation and generally occurs in low concentrations in porewater (Howarth et al. 1983; Luther et al. 1985). Upon heating and acidification it decomposes to mostly S^0 and SO_3^{2-}. Thiosulfate is, accordingly, not distilled in hot HCl ($< 0.5\%$) and is only inefficiently distilled in hot HCl with Cr^{2+} (30–35%). This was shown with radioactive thiosulfate and was the case irrespectively of whether the inner or the outer sulfur atom was labelled (Jørgensen & Fossing, unpublished). Organic sulfur compounds seem not to be reduced by Cr^{2+} (Zhabina & Volkov 1978; Howarth & Jørgensen 1984; Canfield et al. 1986).

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