Pathways and Microbiology of Thiosulfate Transformations and Sulfate Reduction in a Marine Sediment (Kattegat, Denmark)†

BO BARKER JØRGENSEN* AND FRIEDHELM BAK

Department of Ecology and Genetics, University of Aarhus, Ny Munkegade, DK-8000 Aarhus C, Denmark

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Reductive and oxidative pathways of the sulfur cycle were studied in a marine sediment by parallel radiotracer experiments with $^{35}$SO$_4^{2-}$, H$_2$S$_8$, and $^{35}$S$_2$O$_3^{2-}$ injected into undisturbed sediment cores. The distributions of viable populations of sulfate- and thiosulfate-reducing bacteria and of thiosulfate-disproportionating bacteria were concurrently determined. Sulfate reduction occurred both in the reducing sediment layers and in oxidized and even oxic surface layers. The population density of sulfate-reducing bacteria was $\times 10^6$ cm$^{-3}$ in the oxic layer, high enough that it could possibly account for the measured rates of sulfate reduction. The bacterial numbers counted in the reducing sediment layers were 100-fold lower. The dominant sulfate reducers growing on acetate or H$_2$ were gas-vacuolated motile rods which were previously undescribed.

The products of sulfide oxidation, which took place in both oxidized and reduced sediment layers, were 65 to 85% S$_2$O$_3^{2-}$ and 35 to 15% SO$_4^{2-}$. Thiosulfate was concurrently oxidized to sulfate, reduced to sulfide, and disproportionated to sulfate and sulfide. There was a gradual shift from predominance of oxidation toward predominance of reduction with depth in the sediment. Disproportionation was the most important pathway overall. Thiosulfate disproportionation occurred only as cometabolism in the marine acetate-utilizing sulfate-reducing bacteria, which could not conserve energy for growth from this process alone. Oxidative and reductive cycling of sulfur thus occurred in all sediment layers with an intermediate “thiosulfate shunt” as an important mechanism regulating the electron flow.

The microbiology of dissimilatory sulfate reduction and its ecological significance for sulfur cycling in marine sediments have been intensively studied over the past 15 years. New discoveries which show that the pathways of the cycle and the metabolic capacities of the bacteria are much more flexible than has been expected continue to appear. For example, the sulfate-reducing bacteria (SRB) are not bound to reducing environments but may live under oxidizing conditions, in which they may respire with nitrate or even with oxygen (13, 33). There is also recent evidence that sulfate reduction may proceed under highly oxic conditions (10). SRB may also use sulfitc, thiosulfate, or elemental sulfur, either as alternative electron acceptors or for disproportionation of sulfur compounds (a type of inorganic fermentation) in their anaerobic energy metabolism (4, 5, 43). During the disproportionation of thiosulfate, the two sulfur atoms are transformed into sulfate and sulfide. Finally, experiments with washed cell suspensions have established that some SRB are even able to reverse their sulfur metabolism and oxidize reduced sulfur ($S_{\text{red}}$) species such as sulfide or thiosulfate to sulfate in the presence of oxygen (13).

Compared to these rapid advances in our understanding of the ecology and physiology of SRB, the microbiology and pathways of the oxidative part of the sulfur cycle are still poorly known. On the basis of studies of the depth distributions of inorganic sulfur transformations in sediments, it has been concluded that sulfide oxidation generally takes place in the anoxic zone (19, 24). Sulfide oxidation even occurs under strongly reducing conditions in deeper parts of sediments (16, 26). The electron acceptors in the reducing sediment layers have not been quantitatively identified but probably include oxidized manganese and iron minerals (1, 2).

Thiosulfate may be formed by biological sulfide oxidation or by chemical oxidation of free and iron-bound sulfides (7, 18, 34). Thiosulfate concentrations range from low values of $<1$ to 10 $\mu$M in sediments to high values of 100 $\mu$M in salt marshes (32). Recently, thiosulfate has been found to be the most important product of sulfide oxidation in sediments, so its further transformations are therefore key reactions in the sulfur cycle (25, 26). A “thiosulfate shunt” seems to exist in the sulfur cycle of both freshwater and marine sediments. Because of the diverse capabilities of SRB both to reduce sulfate and thiosulfate and to disproportionate sulfur compounds, these bacteria may play a decisive role in the regulation of the electron flow in the sulfur cycle.

We traced the main pathways of SO$_4^{2-}$, H$_2$S, and S$_2$O$_3^{2-}$ transformations in a marine sediment and studied the viable populations of SRB. The goal was to reach an integrated understanding of the physiological capabilities of the bacteria relative to their activities in the sulfur cycle. The concurrent transformations in whole sediment cores were monitored for four radiotracers: SO$_4^{2-}$, H$_2$S, and S$_2$O$_3^{2-}$ with either the inner (sulfonate) or the outer (sulfane) sulfur atom labeled. The distributions of SRB, thiosulfate-reducing bacteria, and disproportionating bacteria were studied by improved viable counting techniques, and the bacteria were partly characterized.

MATERIALS AND METHODS

Samples. Sediment samples were obtained during a cruise with RV Gunnar Thorson (Danish National Agency for Environmental Protection) in April 1989. Sediment cores with intact vertical zonations were collected with a Hapscorer (28) at station 1243 (56°25'N, 11°55'E) in southern
Kattegat, Denmark. The station is situated at the transition between the Baltic Sea and the North Sea at a water depth of 33 m. Subcores were sampled on deck with 26- and 36-mm inner diameter Plexiglas tubes, some of which were supplied with 1-mm-wide silicone-sealed holes for injection of the radiotracer. To avoid oxygen gradients in the overlying water and thereby impede oxygen flux into the sediment during incubation, we siphoned off the water from the cores to leave a film only a few millimeters thick over the sediment. The cores were kept at the in situ temperature of 5.5°C for immediate radiotracer experiments on board the ship or for microbiological studies in the laboratory 4 days later.

Radiotracer experiments. Studies of sulfur transformations were done on different sediment cores with four different 35S-labeled tracers: SO4^2-, H2S, and FeS2^- with either the inner or the outer sulfur atom labeled. The tracers were injected horizontally into intact sediment cores at 1-cm depth intervals. After incubation at the in situ temperature for 3, 7, or 18 h, the cores were cut into 1- or 2-cm segments and fixed with ice-cold ZnCl2 (see below) for later determination of 35S distributions in sulfate, thiosulfate, or Sred (Sred = HS^- + FeS + S0 + FeS2). Duplicate cores were analyzed for each tracer and incubation period. Details of the radiotracer techniques are described elsewhere (16, 26).

[35S]sulfate reduction. Carrier-free 35SO4^-2 (2 μl with <0.1 nmol of SO4^-2; 80 kBq; Isotope Laboratory, Risø, Denmark) was injected (22). After incubation, samples were fixed in 5 ml of cold 20% ZnCl2. The formation of reduced 35S was analyzed by a single-step chromium reduction method in which all Sred is converted into H2S and trapped as ZnS (14, 41a, 47). In some cores, reduced 35S was analyzed separately in pools of acid volatile sulfide (AVS; mostly FeS), S0, and FeS2 (39). Elemental sulfur was first extracted with CS2 from fixed samples. AVS was subsequently solubilized as H2S in an acid solution at room temperature. By a final boiling with Cr^3+ in acid, FeS2 was extracted. Sulfate reduction rates were calculated as described by Jørgensen (22).

[35S]sulfide oxidation. To trap radiolabeled thiosulfate produced during incubation by the oxidation of [35S]sulfide, we injected a nonradioactive solution of thiosulfate (three injections of 50 μl of 15 mM Na2S2O3 at 1-cm depth intervals) into the cores. The sediment was preincubated for 1 h while the thiosulfate gradually dissipated and approached a mean concentration of 0.5 mM. The sulfide tracer was prepared from 35S-labeled S0 (Amersham), dissolved in acetonitrile, was added to H2S/S0 (Cr^3+) in an acid solution, and the tracer was trapped under N2 in 1 ml of 0.01 N NaOH. The labeled sulfide was immediately injected (20 μl; 5 μM; 100 kBq per injection).

After incubation, samples were fixed in 5 ml of cold 0.2% ZnCl2 which contained 25 mM S04^-2 as a carrier. The fixed samples were centrifuged, and a 100-μl subsample was taken from the supernatant for the direct determination of 35S in all dissolved oxidation products. Another 100-μl subsample was injected into an ion chromatograph for separation of the 35S-labeled oxyanions. Only peaks of S04^-2 and SO4^-2 were detectable at an instrument sensitivity of 1 μM for 50-fold-diluted samples. These peak fractions were collected, and their radioactivities were determined. As a control of radioisotope recovery among the sulfur oxyanions, the sum of 35S04^-2 and 35SO4^-2 accounted for 90 to 105% of the radioactivity of 35S in all dissolved oxidation products (obtained from the direct determination), indicating that other, undetected oxyanions could not carry significant amounts of radioactivity. The amount of 35S remaining in Sred pools was determined by single-step chromium distillation of Sred (14) and determination of the radioactivity in the trapped ZnS. In some samples, separate determinations of 35S in AVS, S0, and FeS2 were made (20, 39).

[35S]thiosulfate transformations. Injections were done in two sets of cores with 35SO4^-2 (5 μl of 10 mM Na2S2O3; 80 kBq; Amersham) in which either the inner or the outer sulfur atom was labeled. After incubation, radioisotope separations were done as described for the H2/35S experiments.

Cultivation of bacteria. (i) Medium and growth conditions. Anaerobic sulfur compound-utilizing bacteria were enumerated and cultivated in a bicarbonate-buffered, sulfide-reduced mineral medium containing (per liter) the following: NaCl, 15.0 g; MgCl2 · 6H2O, 2.0 g; CaCl2 · 2H2O, 0.2 g; KCl, 0.5 g; NH4Cl, 0.1 g; KH2PO4, 0.2 g; NaHCO3, 1.75 g; Na2S · 8H2O, 0.18 g; trace element solution, 2 ml (42); and vitamin solution, 1 ml (44). The pH was adjusted to 7.2. The medium was prepared under an O2-free N2 atmosphere as described by Widdel and Pfennig (45). Substrates (electron donors and acceptors) were added from sterile 0.5 to 2 M stock solutions before inoculation. H2 was used in a mixture of CO2 (H2:CO2, 5:1 [vol/vol]) in the gas phases of tubes or bottles sealed with black rubber stoppers (liquid-gas, 2:3 [vol/vol]). The Hungate technique was used for gassing of culture vessels. Cultures with H2 were incubated horizontally to obtain a large surface to facilitate gas exchange. All cultures received about 100 μM Na2S2O3 as an additional reducing agent prior to inoculation. All incubations were done at 20°C in the dark. Growth tests were conducted with 20-ml screw-cap- or stopper-sealed tubes. Growth was monitored by measuring the turbidity at 500 nm in a spectrophotometer.

(ii) Isolation and maintenance of strains. Pure cultures were obtained by repeated application of deep-agar dilutions as described by Widdel and Pfennig (45). Isolated colonies were checked for purity with microscopic controls and with growth tests in complex medium containing yeast extract (0.25%), pyruvate (5 mM), glucose (5 mM), and fumarate (5 mM). Stock cultures were kept at 4°C in the dark and transferred to fresh medium at monthly intervals.

(iii) Enumeration procedures. Numbers of viable sulfate- or thiosulfate-utilizing bacteria were estimated with most-probable-number (MPN) dilutions in liquid medium (3). The dilution series were prepared as described by Bak and Pfennig (5b). Samples containing sulfite but no electron donors served as controls. The counting tubes were incubated at 37°C in the dark for a maximum of 4 months and checked every week for growth. Grown cultures were tested for H2/S0 production, and cultures expected to contain thiosulfate-disproportionating bacteria were also checked for SO4^-2 formation. Three different sets of enumerations were made: (i) enumeration of H2-oxidizing SRB or thiosulfate-reducing bacteria with the substrates H2 (1.0 mM acetate as the carbon source), 5.0 mM SO4^-2, and 5.0 mM S03^-2; (ii) enumeration of acetate-oxidizing SRB or thiosulfate-reducing bacteria with the substrates 10.0 mM acetate, 5.0 mM SO4^-2, and 5.0 mM S03^-2; and (iii) enumeration of thiosulfate-disproportionating bacteria with the substrate 10.0 mM S03^-2 (1.0 mM acetate as the carbon source). Enumerations were carried out for four depth intervals: 0 to 1, 1 to 2, 2 to 4, and 4 to 6 cm.

Chemical determinations. (i) Pore water. Pore water from the sediment was obtained by pressure filtration through 0.45-μm-pore membrane filters under N2. Sulfate was determined by nonsuppressed ion chromatography (Waters). Samples for sulfide determinations were collected without
contact to the atmosphere in 0.2% ZnCl₂, and the amount of trapped ZnS was determined by the spectrophotometric methylene blue method (9).

(ii) Sediment. Elemental sulfur was extracted with CS₂ from ZnCl₂-fixed sediment samples and analyzed spectrophotometrically after cyanalysis and complexation with iron (40). The amounts of AVS and chromium-reducible sulfide were determined from the ZnS traps after distillation of the radiolabeled pools described above. The amount of ZnS was determined by the methylene blue method (9) after appropriate dilutions. The FeS₂ concentration was calculated by difference from chromium reducible sulfur (CRS) minus S⁰ and AVS.

(iii) Cultures. The amount of sulfate in bacterial cultures was determined by the turbidimetric BaSO₄ precipitation method (37). Sulfide was analyzed photometrically after reaction with a CuSO₄·HCl reagent by rapidly measuring the light extinction at 480 nm caused by the CuS precipitate (11).

RESULTS

Sediment. The sediment consisted of sandy and silty mud with a mean organic content of 4.4% (dry weight). Oxygen penetrated to a depth of 9 mm below the sediment surface (35a). The sediment was brownish and oxidized in the upper 0 to 2 cm, below which the color shifted to black or dark grey. Free sulfide was not detectable (<1 μM) in the pore water of the upper 0 to 10 cm. The sediment and the overlying seawater had a temperature of 5.5°C, and the salinity was 32.4‰. The oxygen concentration of the seawater was 80% of air saturation.

Sulfate reduction. The depth distribution of sulfate reduction rates is shown in Fig. 1A. The highest rates, 16 to 19 nmol of SO₄²⁻ cm⁻³ day⁻¹, were found in the reduced sediment layers below 2 cm. Significant activity of the SRB was, however, also observed in the suboxic zone at a 1- to 2-cm depth and even in the oxic zone at a 0- to 1-cm depth.

Sulfate reduction could therefore be detected from the ³⁵S incubations only because the reduced label was trapped in elemental sulfur and pyrite. Only below 2 cm was a part of the labeled sulfide recovered from the AVS fraction. On average, for the 0- to 10-cm depth interval studied, 31% of the reduced label was found in AVS, 33% was found in S⁰, and 36% was found in FeS₂.

AVS accumulated in the sediment below the oxidized zone. Elemental sulfur constituted a small fraction of the particulate sulfur compounds at all depths, while pyrite was quantitatively the dominant sulfur pool. The sulfide concentration had a slight peak at a 2-cm depth, below which the concentration gradually decreased (Fig. 1B). The peak coincided with the redox transition at a 2-cm depth, which was, as expected, the zone in which the most intensive oxidation of S_red compounds to sulfate took place.

Sulfide oxidation. The maximal degree of [³⁵S]sulfide oxidation, 46% of the injected tracer, was found near the redox transition at a 2-cm depth (Fig. 2A). [³⁵S]sulfide oxidation was less intense in the oxic zone, but even in the reduced sediment it proceeded down to a 10-cm depth. Thiosulfate was the main product, constituting up to 85% of the oxidized ³⁵S products after 3 h of incubation (Fig. 2B). The most efficient oxidation to sulfate was found in the oxic surface layer. During prolonged incubation of up to 18 h, some of the thiosulfate was oxidized further to sulfate.

Thiosulfate transformations. The time course of [³⁵S]thiosulfate transformation to ³⁵SO₄²⁻ and ³⁵S_red in the sediment cores is shown in Fig. 3 for the uppermost 0 to 1 cm. The rates of thiosulfate depletion were constant and similar in experiments with the inner or the outer sulfur atom labeled.
The $^{35}$S-labeled products of thiosulfate differed strongly, however, between the two sets of experiments. When the inner sulfur atom was labeled (Fig. 3A), the $^{35}$S-labeled products were 16% $\text{S}_{\text{red}}$ and 84% $\text{SO}_4^{2-}$. Since a conversion of the oxidized, inner sulfur atom to sulfide represents a net reduction, 16% of the thiosulfate was reduced. When the outer sulfur atom was labeled (Fig. 3B), the $^{35}$S-labeled products were 76% $\text{S}_{\text{red}}$ and 24% $\text{SO}_4^{2-}$. Since a conversion of the reduced, outer sulfur atom to sulfate represents a net oxidation, 24% of the thiosulfate was oxidized. The remaining 60% $[100 - (16 + 24)]$ of the thiosulfate was metabolized, not by net reduction to sulfide or net oxidation to sulfate, but by disproportionation concurrently to sulfide and sulfate (24, 26).

A larger fraction of thiosulfate was transformed in the oxic zone than in the reduced sediment (Fig. 4). The formation of $^{35}$S$_{\text{red}}$ and $^{35}$SO$_4^{2-}$ from thiosulfate with the inner and outer sulfur atoms labeled also changed with depth in the sediment. The formation of $^{35}$SO$_4^{2-}$ decreased with depth with both tracers, while the formation of $^{35}$S$_{\text{red}}$ increased.

Like the calculations from the time course experiments, the data in Fig. 4A and B were used to calculate the depth distributions of thiosulfate reduction, oxidation, and disproportionation. The total rates of thiosulfate transformation decreased threefold between the surface and the reducing zone (Fig. 5A). The relative contribution of thiosulfate reduction increased strongly with depth (Fig. 5B). The distribution of thiosulfate oxidation was similar to that of sulfide oxidation, with a maximum near the redox transition at a 2-cm depth. Disproportionation decreased slightly with depth and was overall the most important pathway of thiosulfate transformation.

**Bacteria.** All bacteria observed during the enumerations were SRB. Other dissimilatory sulfur compound-utilizing bacteria did not occur. In some cases, it took up to 3 months for the count to fully develop. The total numbers of SRB recorded (on $\text{H}_2$, acetate, and thiosulfate) are shown in Fig. 6, together with the sulfate reduction rates. Maximum population densities of SRB, $2 \times 10^6$ cells cm$^{-3}$, were surprisingly found in the uppermost 0 to 2 cm, where the sediment...
was oxidized and the measured sulfate reduction rates were relatively low.

**H₂-utilizing SRB.** The numbers of H₂-oxidizing SRB decreased from 1.1 x 10⁹ cells cm⁻³ in the uppermost layer to about 100-fold lower numbers at a 4- to 6-cm depth (Fig. 7). Two morphological types dominated: small, mostly motile *Desulfovibrio* species (Fig. 8A, arrow) and relatively large, motile rods with gas vacuoles (Fig. 8B). The latter species were numerically dominant but occurred only in MPN counts from the upper 0 to 2 cm, whereas *Desulfovibrio* species were present at 10⁴ to 10⁵ cells cm⁻³ at all depths. Four representative *Desulfovibrio* strains and one gas-vaculated SRB (strain WK1) were isolated in pure cultures and tested for sulfate and thiosulfate utilization. All five strains reduced sulfate or thiosulfate to sulfide with H₂ as the electron donor. None of the strains was able to disproportionate thiosulfate or to grow on H₂ in the absence of sulfate or thiosulfate. The gas-vaculated strain, WK1, isolated from the highest growth-positive dilution of the H₂ enumeration series, was moderately psychrophilic. The strain grew well at 19°C but not at 25°C. Strain WK1 efficiently utilized H₂, lactate, malate, and pyruvate as electron donors for sulfate reduction. Older cultures of the *Desulfovibrio* species often formed coccoid bodies (Fig. 8A), especially upon prolonged storage.

**Acetate-utilizing SRB.** The numbers of SRB growing on acetate were highest in the 0- to 2-cm interval and decreased significantly in deeper layers (Fig. 7). Oval or slightly curved...
cells, morphologically resembling SRB of the genus Desulfo-
bacter or Desulfobacterium, developed. In the highest
growth-positive dilutions of counts from the 0- to 1- and 1- to 
2-cm zones, bacteria containing gas vacuoles were again 
observed (Fig. 8C). Most SRB, which developed in enumer-
ations with acetate, had the tendency to grow in chains or
dense clumps. Higher dilutions sometimes showed weak but
significant H2S production (1 to 2 mM) without an increase in
turbidity. Upon careful observation, however, a thin bacte-
rial film or small colonies on the glass wall were detectable,
mainly in the bottom portions of the culture tubes. The bac-
teria within the film morphologically resembled smaller 
SRB of the genus Desulfo bacter or Desulfobacterium. In 
one of the tubes from the 2- to 4-cm interval, an undescribed,
thick, spirilloid SRB developed (Fig. 8D).

Two Desulfobacter-like species from higher dilutions (0-
to 1- and 2- to 4-cm depths) were subcultured and tested for 
sulfur compound utilization. With 15 mM acetate as the
electron donor, both cultures reduced sulfate and thiosul-
fate. In the presence of only small amounts of acetate (1 
mM), both cultures also disproportionated thiosulfate. Thio-
sulfate disproportionation was, however, not coupled to
growth.

Thiosulfate-disproportionating SRB. The estimated num-
bers of thiosulfate-disproportionating bacteria were about 
10-fold lower than were the numbers of H2 or acetate-
utilizing SRB at all depths (Fig. 7). With one exception (one 
tube of a 10−1 dilution from the 0- to 1-cm depth), only a
small fraction (10 to 20%) of the added thiosulfate was 
disproportionated and significant growth could not be ob-
served. Only small flocs or clumps consisting of Desulfobac-
ter- or Desulfobacterium-like SRB were observed in the 
bottom portions of sulfate-positive tubes. When the bacteria 
were transferred into fresh medium, growth remained weak 
unless the amount of acetate was increased.

**DISCUSSION**

**Sulfate reduction.** The depth distribution of sulfate reduc-
tion showed a typical rate maximum in the uppermost part 
of the black, reduced sediment (Fig. 1A). There was, however,
also rather high sulfate reduction in the oxidized (Eh > 0 mV) 
sediment above a 2-cm depth and even in the oxic (O2-
containing) sediment above a 0.9-cm depth. Similar obser-
vations of sulfate reduction in oxidized surface layers have 
been made in other marine and freshwater sediments (5b, 
21). In the absence of free sulfide or AVS in the oxidized 
surface sediment, the radioactivity of the H2S formed was 
retained in the less reactive sulfur compounds, elemental 
sulfur and pyrite (8, 39). Because of the oxidizing condi-
tions, a significant but unknown fraction of the sulfide formed 
from sulfate reduction may have been rapidly reoxidized within 
the uppermost 1 to 2 cm; thus, the measured sulfate reduc-
tion rates may have been underestimated.

The potential for high sulfate reduction under oxic condi-
tions was emphasized by the observation of about 50-fold-
higher population densities (MPN counts) of SRB at the 0- to 
1-cm depth than at the 3- to 5-cm depth (Fig. 6). There are 
several possible explanations for this distribution. The SRB 
may be able not only to tolerate oxygen for prolonged 
periods (12) but also perhaps to carry out sulfate reduction in 
the presence of oxygen. The latter has been observed in 
O2-supersaturated surface layers of photosynthetically 
active cyanobacterial mats (10). It is also possible that the SRB 
in the surface sediment are clustered within anoxic and 
reducing microenvironments (21). Because of diffusive and 
metabolic constraints, however, such microenvironments 
would be expected to have a characteristic size of several 
tens or hundreds of micrometers. Although they should thus

![FIG. 6. Total numbers of SRB, as determined by the MPN technique, on acetate or H2 substrate at different depths in Kattegat sediment. The concurrently measured sulfate reduction rates are shown for comparison. The depth of oxygen penetration into the sediment (0.9 cm) and the transition depth between the brown, oxidized surface sediment and the black, reduced sediment are shown. d, Day.](image-url)

![FIG. 7. Depth distributions of H2-utilizing, acetate-utilizing, and thiosulfate-disproportionating SRB, as determined by the MPN technique.](image-url)
be easy to recognize during measurements with oxygen microelectrodes, we have not encountered such microenvironments during our studies. Finally, a versatile metabolism has been demonstrated among the SRB in recent years, including respiration with nitrate or even oxygen (13, 33). Although such respirations could help to explain the observed bacterial distribution, the quantitative importance of these metabolic capabilities in sediments is still unknown.

Sulfate oxidation. Oxidation of sulfide was directly demonstrated by the use of H$_2^{35}$S in both the oxidized surface sediment and the black, reduced sediment at a 2- to 10-cm depth (Fig. 2A). A comparison of the high sulfate reduction rates and the slow accumulation of S$_{red}$ in both zones similarly showed that intensive reoxidation of sulfide must have taken place. Sulfate reoxidation in the upper 0 to 10 cm was 2.15 mmol m$^{-2}$ day$^{-1}$. The mean accumulation of S$_{red}$ with depth was 4 μmol of S cm$^{-2}$ per cm or 40 μmol of S cm$^{-2}$ over a 0- to 10-cm depth (Fig. 1B, broken line). With a mean sediment accumulation rate of 1 mm year$^{-1}$ for the area (31a), the rate of sulfide burial over the top 10 cm was 0.11 mmol m$^{-2}$ day$^{-1}$. Thus, only 2% ([0.11 × 100]/2.15) of the sulfide produced from sulfate reduction was retained as FeS$_2$, FeS, and S$^0$. The remaining 95% was reoxidized. Since there was no detectable sulfide in the pore water, reoxidation cannot be explained by diffusion of sulfide up to the oxic and oxidized surface layers. It is therefore in accordance with these mass balance considerations that reoxidation of sulfide was found to take place throughout the sediment column.

Anoxic reoxidation of $^{35}$S]sulfide also has been directly demonstrated in other sediments from both marine and freshwater localities (16, 26). The degree of reoxidation varies from between 60 and 97% of all sulfide produced from sulfate reduction in the area between the Baltic Sea and the North Sea (27). In Lake Constance sediment, 98% of the sulfate used by the SRB was derived from reoxidized sulfide and only 2% was derived from "new" sulfate diffusing down from the overlying lake water (5b, 5c). The oxidants for anoxic sulfide oxidation have not been directly identified. However, oxidized iron and manganese minerals may play an important role (1, 2, 24, 29).

The addition of nonlabeled thiosulfate, which was necessary to transiently trap $^{35}$S$_2$O$_3^{2-}$ formed from $^{35}$S]sulfide, was not expected to have changed the relative pathways of sulfide oxidation. Thiosulfate addition increased the rate of sulfide formation, especially from disproportionation in the oxidized zone. The increase was, however, less than twofold throughout most of the sediment and could therefore not have changed the chemistry of the sediment significantly during the short incubations.

It was an experimental problem in our earlier radiotracer studies of sulfide oxidation that only a small fraction of the radiolabel was found in more oxidized sulfur compounds, even in the oxidized surface layers of the sediment. The reason for this was that the radiolabel was rapidly transferred from H$_2^{35}$S into other reduced, but unreactive, sulfur

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**FIG. 8.** Photomicrographs of undescribed SRB observed in MPN enumerations. Bars: 10 μm in A and C and 5 μm in B and D. (A) Coccoid bodies observed in a 2-week-old culture of an H$_2$-oxidizing *Desulfovibrio* sp. The arrow shows an intact *Desulfovibrio* cell. (B) Strain WK1, an H$_2$-oxidizing SRB with gas vacuoles. The arrow shows a spherical cell which had just exploded. (C) SRB with gas vacuoles observed in enumerations with acetate. (D) Acetate-utilizing, sulfate-reducing spirillum.
pools, such as $\text{S}^0$ or FeS, by isotopic exchange (15, 16a). The specific radioactivity of the reacting sulfide thereby became very low and the radiotracer transformations could not be used for calculations of the amount of sulfide oxidized. An oxidation of up to 46%, as found in the present study (Fig. 2A), is the highest that we have recorded so far. The factors controlling isotopic exchange are not quantitatively understood, but the presence of polysulfides seems to be highly catalytic for the exchange (15).

Isotopic exchange may also be an important mechanism for the formation of $^{35}$S-labeled $\text{S}^0$ and perhaps FeS$_2$ in the oxidized surface sediment during sulfate reduction measurements (Fig. 1A). Isotopic exchange into more stable sulfur pools is thus a mechanism which transiently preserves $^{35}$S in reduced form and helps to reveal in situ rates of sulfate reduction despite rapid reoxidation of the H$_2$S formed.

**Thiosulfate transformations.** The rates of thiosulfate depletion were similar in the experiments with the inner or the outer sulfur atom labeled (Fig. 3), indicating, as should be expected, that the two radioactive thiosulfate pools were similarly metabolized by the bacteria and that isotopic exchange reactions, which may occur between the outer sulfur atom and sulfide at higher temperatures (41), were not of significance. Thiosulfate was the main product of sulfide oxidation throughout the sediment (Fig. 2), in accordance with radiotracer results from other marine and freshwater sediments (16, 26) and with observations that thiosulfate is one of the main products from the chemical oxidation of H$_2$S, FeS, and FeS$_2$ (7, 18, 34).

Thiosulfate reduction occurred throughout the sediment but became a relatively more important pathway in the deeper layers (Fig. 5). All of the SRB strains from the sediment could also reduce thiosulfate. Bacteria utilizing thiosulfate but not sulfate, e.g., *Campylobacter* spp., were not observed. Consequently, SRB appeared to be the principle thiosulfate-reducing bacteria in the sediment. Thiosulfate is generally a preferred electron donor in these bacteria because, unlike sulfate, it does not require ATP-consuming activation. The addition of thiosulfate to freshwater sediment slurries has accordingly been found to significantly inhibit sulfate reduction (26).

The oxidation of thiosulfate to sulfate in all sediment layers, with a maximum at the redox transition, is consistent with the observation of a similar distribution of sulfide oxidation (Fig. 5). About 50% of the thiosulfate in the 0 to 10 cm layer was oxidized, but 100% was oxidized in the surface layer. The LS and SO$_4^{2-}$ were oxidized, reduced, and transformed to SO$_4^{2-}$, while the reduced, outer sulfur atom was transformed to H$_2$S. An H$_2$S-S$_2$O$_3^{2-}$ cycle in which half of the sulfur atoms remained at the oxidation state of $\sim 2$ was thus established. Since thiosulfate was the main product of sulfide oxidation, this internal recycling of sulfur is quantitatively an important pathway of the sulfur cycle. Such a thiosulfate shunt has now been found in a range of marine and freshwater sediments (16, 26).

**Viable counts of SRB.** The population sizes of SRB in the sediment were studied by an MPN technique. Incubations were done at an ecologically realistic temperature, 19°C, and the growth of bacteria was checked weekly for up to 4 months at the highest dilutions. During recent studies with freshwater sediment, the MPN enumeration technique used here proved to be more efficient (i.e., yielded higher recoveries of SRB) than previously applied techniques, such as deep-agar dilution with complex media and an Fe$^{3+}$ indicator for sulfide production (5b). With the MPN technique it is possible to overcome the problem inherent in the deep-agar method, in which fast-growing colonies tend to outgrow more slowly growing organisms, which are, as expected, more abundant, and to blacken the agar. In the MPN technique, even bacteria with doubling times of several days may eventually be recognized. Denitrifiers of up to $10^{10}$ SRB cm$^{-3}$ were found in the oxidized sediment zone (Fig. 6). These densities are 1 to 3 orders of magnitude higher than those previously reported for SRB from marine sediments (16, 23).

A comparison of the measured population sizes of SRB and the measured in situ rates of sulfate reduction indicated that the bacteria were respiring at specific rates of $3 \times 10^{-15}$ to $5 \times 10^{-15}$ mol cell$^{-1}$ day$^{-1}$ in the upper 0 to 2 cm. These rates are in the same range as the calculated specific respiration rates for SRB growing in pure cultures on H$_2$, lactate, or pyruvate: $0.2 \times 10^{-15}$ to $50 \times 10^{-15}$ mol cell$^{-1}$ day$^{-1}$ (23). The bacterial densities are thus high enough to potentially account for their metabolism in the present study. The specific respiration rates below 2 cm were 100-fold lower, while those calculated from several earlier studies were about 1,000-fold lower. We therefore conclude that the applied MPN technique has improved the viable counting techniques for SRB in sediments by 10 to 1,000 fold. The cell numbers determined are still highly underestimated in the deeper sediment layers, but in the surface sediment the cell numbers are approaching a realistic range as compared with the measured activity of the bacteria, making the viable counts much more interesting with respect to characterization of the different species of SRB. Furthermore, the types of bacteria that develop in the MPN tubes are different from those that develop in the deep-agar tubes. We thus conclude that these viable counting techniques cannot be used for the quantification of natural populations of SRB and for their physiological characterization. It is necessary that counting techniques and media that allow the bacteria to grow under ecologically realistic conditions are established. Bak and Pfennig (5b) found similarly high recoveries of SRB in freshwater sediments.

**SRB.** It was an important new result that gas-vacuolated types were the most abundant SRB in the upper 0 to 2 cm of the sediment. These organisms dominated in the highest positive dilutions of the MPN counts, both with acetate and with H$_2$ as electron donors. In marine enrichment cultures of SRB, which are incubated at low temperatures (4 to 5°C), such gas-vacuolated types often develop (20a). Similarly at lower dilutions on H$_2$ did the classical, fast-growing *Desulfovibrio* species dominate in abundances which were similar to those reported in earlier studies (17, 23). Species with gas vacuoles must thus be among the dominant SRB in theoxic and oxidized layers of the sediment. So far, only one strain containing gas vacuoles has been tentatively named *Desulfo bacterium* vacuolatum (43), but this strain has not yet been described.

Although morphologically clearly different from all SRB so far described, strain WK1 resembled physiologically the classical H$_2$-oxidizing *Desulfovibrio* species. A further unusual feature was the formation of spherical structures in cultures of strain WK1 (Fig. 8B). These structures were either free swimming or attached to cells of strain WK1. The formation of coccolid bodies in older cultures of the *Desulfovibrio* species (Fig. 8A) was a similar phenomenon and is currently being studied.

Several other undescribed SRB morphologies, e.g., the spirilla shown in Fig. 8D, developed in the acetate MPN tubes. Moreover, acetate-oxidizing SRB were at least as abundant as H$_2$-oxidizing SRB. This result is in contrast to
those for freshwater sediments, in which SRB physiologically resembling the H₂-oxidizing Desulfovibrio species were more abundant, or to those for Wadden Sea sediments, in which lactate oxidizers (and probably the classical Desulfovibrio species) were found in the highest numbers (5b, 31). Because of the lower recovery in earlier studies, however, these relative distributions may be influenced by the selectivity of the applied counting procedures as much as by the actual abundances of the physiological types of SRB.

The present study thus demonstrates directly for the first time that acetate-oxidizing SRB dominate in marine sediments. A predominance of acetate oxidizers is consistent with observations of the compositions of fatty acid biomarkers of SRB in marine sediments (38). It is also in accordance with repeated demonstrations of acetate as the main electron donor for sulfate reduction in marine sediments (e.g., 6, 35, 36, 46).

Thiosulfate-disproportionating bacteria. Thiosulfate-disproportionating bacteria were counted by an anaerobic MPN technique with thiosulfate as the energy substrate and a small amount of acetate as the carbon source. The addition of acetate at a low concentration was necessary to promote the growth of these chemolithoheterotrophic bacteria (5). To discriminate them from acetate-oxidizing SRB with S₂O₃²⁻ as an electron acceptor, we found it necessary to check for the simultaneous production of SO₄²⁻ and H₂S.

The anaerobic, thiosulfate-disproportionating bacteria isolated from the marine sediment were distinctly different from the types that have been found in freshwater sediments (5b). In contrast to the freshwater strains, the marine strains appeared to be unable to grow by S₂O₃²⁻ disproportionation alone. Obviously, S₂O₃²⁻ was only cometabolized by the bacteria which grew marginally by acetate oxidation coupled to thiosulfate reduction. No specialized disproportionating bacteria, such as Desulfovibrio sulfodismutans, which is commonly present in enrichments from freshwater sources, were found (5). It seems that marine bacteria cannot couple the disproportionation of sulfur compounds to energy conservation. This idea is consistent with pure-culture studies which demonstrated that marine SRB are unable to grow by disproportionation reactions (30). In enrichments from freshwater sediments, only H₂-utilizing SRB could disproportionate thiosulfate (5a). The reason for these differences between marine and freshwater strains is yet unexplained.

It was found in the present study that all of the SRB were able to use S₂O₃²⁻ as an electron acceptor and that all of the S₂O₃²⁻-reducing bacteria were SRB. The depth distributions of SRB and thiosulfate-reducing bacteria were thus identical. It was furthermore demonstrated that SRB, which can disproportionate thiosulfate, may carry out this reaction concurrently with the reduction of thiosulfate in the presence of suitable electron donors (30). It has also been shown recently that some SRB are able to oxidize sulfide and thiosulfate during aerobic respiration in the presence of oxygen (13). It is therefore likely that in large groups of bacteria which carry out the different oxidative and reductive transformations of sulfur compounds in the sediment. In conclusion, it appears that SRB have a very broad metabolic capacity and that they may play a much more diverse role in sediments than we have previously thought.

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