RESEARCH ARTICLE

# Anaerobic sulfur oxidation in the absence of nitrate dominates microbial chemoautotrophy beneath the pelagic chemocline of the eastern Gotland Basin, Baltic Sea

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## Abstract

Oxic–anoxic interfaces harbor significant numbers and activity of chemolithoautotrophic microorganisms, known to oxidize reduced sulfur or nitrogen species. However, measurements of *in situ* distribution of bulk carbon dioxide ($CO_2$) assimilation rates and active autotrophic microorganisms have challenged the common concept that aerobic and denitrifying sulfur oxidizers are the predominant autotrophs in pelagic oxic–anoxic interfaces. Here, we provide a comparative investigation of nutrient, sulfur, and manganese chemistry, microbial biomass distribution, as well as $CO_2$ fixation at the pelagic redoxcline of the eastern Gotland Basin, Baltic Sea. Opposing gradients of oxygen, nitrate, and sulfide approached the detection limits at the chemocline at 204 m water depth. No overlap of oxygen or nitrate with sulfide was observed, whereas particulate manganese was detected down to 220 m. More than 70% of the bulk dark $CO_2$ assimilation, totaling 9.3 mmol C $m^{-2}$ $day^{-1}$, was found in the absence of oxygen, nitrite, and nitrate and could not be stimulated by their addition. Maximum fixation rates of up to 1.1 μmol C $L^{-1}$ $day^{-1}$ were surprisingly susceptible to altered redox potential or sulfide concentration. These results suggest that novel redox-sensitive pathways of microbial sulfide oxidation could account for a significant fraction of chemolithoautotrophic growth beneath pelagic chemoclines. A mechanism of coupled activity of sulfur-oxidizing and sulfur-reducing microorganisms is proposed.

## Introduction

Microbial dark carbon dioxide ($CO_2$) fixation occurs in many pelagic oxic–anoxic interfaces. These include marine environments such as Framvaren Fjord, Norway (Mandernack & Tebo, 1999), Cariaco Trench, Caribbean Sea, off Venezuela (Tuttle & Jannasch, 1979), brackish environments such as the Black Sea (Sorokin, 1964), the Baltic Sea (Detmer *et al.*, 1993; Labrenz *et al.*, 2005), or Big Soda Lake, NV (Cloern *et al.*, 1983), as well as freshwater systems such as Lake Kinneret, Israel (Hadas *et al.*, 2001). In these oxic–anoxic interfaces, dark $CO_2$ fixation contributes significantly to the overall inorganic carbon assimilation (e.g. Jørgensen *et al.*, 1991; Detmer *et al.*, 1993; Mandernack & Tebo, 1999; Taylor *et al.*, 2001). Taylor *et al.* (2001) reported that seasonally dark $CO_2$ assimilation could outpace phototrophic primary production in surface waters of the Cariaco Trench. As such, assimilation of inorganic carbon by chemolithoautotrophic microorganisms provides a significant source of nonphototrophic carbon, altering the overall carbon budget and isotopic composition of organic matter in anoxic water and sediments (Taylor *et al.*, 2001).

Nonetheless, it remains unclear which types of microbial energy metabolisms fuel the observed $CO_2$ assimilation.

rRNA gene surveys demonstrated that clades of known sulfur-oxidizing *Gamma-* and *Epsilonproteobacteria* are abundant in oxic–anoxic interfaces of the Black Sea, Cariaco

Trench as well as the anoxic Basins in the central Baltic Sea (Madrid *et al.*, 2001; Vetriani *et al.*, 2003; Labrenz *et al.*, 2004, 2005; Lin *et al.*, 2006), suggesting that mostly aerobic and denitrifying sulfur oxidizers are responsible for the autotrophic activity. Additionally, several strains of sulfur-oxidizing bacteria have been isolated from these interfaces (Tuttle & Jannasch, 1973; Jannasch *et al.*, 1991). However, in most anoxic basins, the level of dark $CO_2$ fixation surpasses the diffusive supply of sulfide, oxygen, and nitrate up to 100-fold compared with stoichiometries of sulfur-oxidizing bacteria (Jørgensen *et al.*, 1991; Taylor *et al.*, 2001; Zopfi *et al.*, 2001). Nonetheless, *in situ* sulfide oxidation rates in the Black Sea and flux-balance calculations on several anoxic basins indicate that significantly more sulfide enters the oxic–anoxic interface and is oxidized than any other reduced inorganic species (ammonium, $Fe^{2+}$, $Mn^{2+}$, or hydrogen) (Jørgensen *et al.*, 1991; Taylor *et al.*, 2001; Zopfi *et al.*, 2001; Neretin *et al.*, 2003; Ho *et al.*, 2004). Moreover, the bulk dissolved inorganic carbon (DIC) assimilation occurs several meters below the zone of available oxygen and nitrate/nitrite in the Black Sea and Cariaco Basin (Jørgensen *et al.*, 1991; Taylor *et al.*, 2001). It has been suggested that either large-scale advective mixing supplies significantly more electron donors and acceptors than indicated by current flux-balance calculations (Jørgensen *et al.*, 1991; Taylor *et al.*, 2001; Zopfi *et al.*, 2001; Ho *et al.*, 2004) or that novel pathways of microbial sulfur oxidation via reduction of particulate metal oxides, such as manganese or iron oxide, could fuel microbial dark $CO_2$ fixation below pelagic chemoclines (depth of first appearance of $H_2S$) (Jørgensen *et al.*, 1991; Taylor *et al.*, 2001).

We have recently shown that a single phylotype closely related to the obligate chemolithoauthotrophic sulfur-oxidizing epsilonproteobacterium *Thiomicrospira denitrificans* constitutes between 8% and 15% of the microbial community at the Gotland Basin redoxcline (Labrenz *et al.*, 2005; Grote *et al.*, 2007). By combining dark $CO_2$ fixation measurements with flow cytometric cell sorting, we further demonstrated that an even higher percentage of microorganisms (about 30%) actively assimilates $CO_2$ (Jost *et al.*, 2008). Recent catalyzed reporter deposition-FISH–microautoradiography and stable isotope probing experiments corroborated these findings and suggested that *Epsilonproteobacteria* constitute up to 70% of the $CO_2$-incorporating cells below the Gotland Basin chemocline, whereas a smaller fraction might consist of *Gammaproteobacteria* (Grote *et al.*, 2008; Glaubitz *et al.*, 2009).

Here, we present a detailed analysis of sulfur, nitrogen, and manganese speciation along the oxic–anoxic interface of the Gotland Basin and investigate the potential of alternative sulfur oxidation pathways as a driving force for *in situ* $CO_2$ fixation activity. We hypothesize that anaerobic manganese-dependent sulfur oxidation by these *Epsilon-* and *Gammaproteobacteria* could fuel microbial chemoautotrophic growth *in situ*.

## Materials and methods

### Study area and sampling

The eastern Gotland Basin is a 248-m-deep basin in the central Baltic Sea and contains one of the largest coherent water masses of the Baltic Sea. Sampling was performed during cruise AL256 aboard R/V '*Alkor*' in May 2005 at the deepest part of the basin (57°19.2′N 19°57′E, water depth between 228 and 242 m). Profiles of temperature, salinity, and oxygen were recorded using a Seabird SBE 911*plus* conductivity–temperature–depth profiler (Sea-Bird Electronics Inc., WA) mounted on a rosette water sampler. Water samples from discrete depths were collected with 5-L free-flow bottles (Hydrobios, Kiel, Germany) attached to the sampler. Subsamples were directly filled into calibrated 120-mL oxygen bottles with at least three flask volumes overflow. Special care was taken to avoid any oxygen contamination during the filling procedure. For control purposes, the filling procedure was repeatedly performed under a stream of argon to avoid any air contact with the water samples. No differences in chemical as well as activity measurements were observed between both procedures.

### Chemical analyses

Analyses of inorganic nutrients, as well as oxygen and sulfide were performed immediately after sampling using standard manual colorimetric methods (Grasshoff *et al.*, 1983). Samples for the total inorganic carbon concentration were taken, preserved with mercury chloride, and determined later using the coulometric SOMMA system (Johnson *et al.*, 1993).

Thiosulfate and sulfite concentrations were determined by HPLC analysis of monobromobimane-derivatized samples (Fahey & Newton, 1987; Rethmeier *et al.*, 1997; Zopfi *et al.*, 2004). Five hundred microliter water samples were taken from the free-flow bottles using a Hamilton syringe, filled into 1.5-mL reaction tubes, and instantly derivatized by the addition of 100 μL monobromobimane reagent. Samples were maintained for 30 min at room temperature in the dark, and the reaction was stopped by acidification with 50 μL of methanesulfonic acid (324 mmol $L^{-1}$). Samples were subsequently stored frozen at −80 °C until HPLC analysis within 3 weeks. The stability of the thiosulfate–bimane complex was reported to be somewhat sensitive to long-term storage (Zopfi *et al.*, 2004). Samples spiked with known amounts of thiosulfate were therefore derivatized and analyzed in parallel and did not exhibit significant decomposition of the thiosulfate–bimane complex within this storage period. Monobromobimane reagent was freshly

prepared each day by mixing equal volumes of HEPES buffer (500 mmol $L^{-1}$ HEPES, 50 mmol $L^{-1}$ Na-EDTA, pH 8) and monobromobimane (Calbiochem, Carlsbad, CA; 45 mmol $L^{-1}$ in acetonitrile). Standards of thiosulfate and sulfite were prepared in serum bottles, flushed with nitrogen, and stored at 4 °C.

Analysis was carried out using a gradient HPLC system (Sykam, Fürstenfeldbruck, Germany). Separation was performed on a reversed-phased column (Adsorbospere OPA HR 5μ, 150 × 4.6 mm; Alltech, Unterhaching, Germany) thermostated to 25 °C at a flow rate of 1.0 mL $min^{-1}$. Eluent A was 0.25% v/v acetic acid (pH 3.5) and eluent B was 100% HPLC gradient-grade methanol (Carl Roth, Karlsruhe, Germany). The gradient used was as follows: 0–0.5 min 10% B, 7 min 12% B, 15 min 30% B, 19 min 30% B, 23 min 50% B, 30 min 100% B, 36 min 100% B, 37 min 10% B, and 44 min 10% B. Fluorescence emission was recorded on a Linear Instruments LC 305 fluorescence detector (excitation 380 nm, emission 480 nm). Sulfite and thiosulfate eluted after 3.3 and 5.9 min, respectively. The reproducibility of standards (1 μmol $L^{-1}$) was better than 5%, and at an injection volume of 100 μL, the detection limit was 50 nmol $L^{-1}$.

Zero-valent sulfur was determined in parallel samples as described by Zopfi *et al.* (2001, 2004) with the following modifications: a 1-mL water sample was immediately fixed with 100 μL 2% w/v $ZnCl_2$ and stored at −80 °C. Sulfur was extracted with 0.1 vol. chloroform for 2 h and 70 μL of the chloroform extract was resuspended in 900 μL methanol and analyzed by HPLC. Standard solutions were prepared in methanol : chloroform (90 : 10) before analysis. HPLC separation was carried out on the HPLC system and column described above with an isocratic eluent of 100% HPLC gradient-grade methanol (Carl Roth). $A_{265\,nm}$ was recorded on a Linear Instruments UVIS 204 detector. Injection volume totaled 100 μL and cyclo-$S_8$ sulfur eluted after 3.1 min. The detection limit was 300 nmol $L^{-1}$, and the reproducibility of 5 μmol $L^{-1}$ standards was better than 5%.

Dissolved and particulate manganese analysis was performed according to Hinrichs *et al.* (2002). Exactly 1 L of the water sample was filtered through polycarbonate membranes (0.45 μm poresize; Millipore, Göttingen, Germany) as soon as possible after sampling. Each filter was subsequently rinsed with 18 mΩ water and stored in sterile plastic Petri dishes in the dark until analysis. For the determination of dissolved manganese, 50-mL subsamples of the filtrate were collected, filled into polyethylene bottles (equilibrated with 2% $HNO_3$ and rinsed with sample filtrate), and acidified by addition of 1 mL redistilled $HNO_3$. Complete acid digestion of filters was carried out in closed polytetrafluroethylene autoclaves at 180 °C in a mixture of $HNO_3$, $HClO_4$, and HF. Analysis of manganese was performed by ICP-OES (Perkin Elmer Optima 3000XL). The detection limits for dissolved and particulate manganese were 0.36 and 0.02 μmol $L^{-1}$, respectively.

Eddy diffusion and settling velocities of manganese particles were calculated using the equations given by Schneider *et al.* (2002) and Neretin *et al.* (2003).

### *In situ* dark $CO_2$ fixation

Over a period of 10 days, multiple profiles throughout the redoxcline (180–220 m water depth) were taken for the determination of $CO_2$ dark fixation rates according to the method of Steemann Nielsen (1952) and Jost & Pollehne (1998). The anoxic sodium [$^{14}$C]-bicarbonate stock solution (250 μCi $mL^{-1}$) was prepared as follows: deionized water (18 mΩ) was filled into serum vials and carefully flushed with argon to remove any oxygen. Weighted sodium [$^{14}$C]-bicarbonate crystals (SA 53.0 mCi $mmol^{-1}$; Hartmann Analytic GmbH, Braunschweig) were added to the serum vials under an argon atmosphere in an anaerobic chamber and dissolved.

Incubations were performed in 120-mL oxygen bottles, which were carefully filled as described above. Forty to 60 μL of an anoxic [$^{14}$C]-bicarbonate stock solution was added using a gas-tight Hamilton syringe, thereby only insignificantly altering the total inorganic carbon concentration of about 2000 μmol $L^{-1}$. Samples were incubated at *in situ* temperature (± 1 °C) in the dark. Incubation was stopped by filtration. Before filtration of the samples, 50-μL subsamples were drawn from all incubations and placed into scintillation vials containing 50 μL ethanolamine to quantify the amount of added [$^{14}$C]-bicarbonate. Samples were then filtered onto polycarbonate membrane filters (0.2 μm pore size; Whatman, via VWR International, Weiterstadt, Germany), exposed to HCl fumes for 30 min, mixed with scintillation cocktail (UltimaGold XR, Packard), and counted on board in a Triathler liquid scintillation counter (Hidex Oy, Turku, Finland) and in the laboratory in a TriCarb 2560 TR/X liquid scintillation counter (Packard). $CO_2$ fixation of control samples immediately fixed with 1 mL formalin (37%) before adding [$^{14}$C]-bicarbonate was 0.0043 μmol C $L^{-1}$ $day^{-1}$ (SD 0.0029 μmol C $L^{-1}$ $day^{-1}$). All incubations were started within 15 min of sample collection and lasted between 22 and 26 h.

### Substrate-spiking experiments

According to the results of *in situ* rate measurements, water samples were taken from above, within, and below the zone of maximal *in situ* $CO_2$ fixation (204, 214, and 226 m water depth, respectively). $CO_2$ fixation was analyzed as described above and parallel samples were immediately spiked with working solutions of the following potential electron donors and acceptors: oxygen, sodium nitrate, manganese oxide,

iron oxide, anthraquinone disulfonate (AQDS), sodium thiosulfate, polysulfide, sodium sulfide, and sodium dithionite. Working solutions (2.4 mmol $L^{-1}$) of sodium nitrate, manganese oxide, iron oxide, AQDS, and sodium thiosulfate were prepared by dilution of concentrated sterile stocks (1 mol $L^{-1}$) into sterile-filtered deionized water, filled into butyl rubber-stoppered serum vials, and flushed with argon for at least 15 min. A stock solution of polysulfide was prepared by short heating of 50 mL sodium sulfide solution (1 mol $L^{-1}$) to 90 °C, after addition of 0.11 mol of elemental sulfur under a nitrogen atmosphere. Working solutions of polysulfide and sodium sulfide were flushed with argon before the addition of concentrated stock solutions. The dithionite working solution was prepared from sodium dithionite crystals directly on board. Oxygen solution was prepared from sterile-filtered air-saturated water. All working solutions were prepared within 4 h before use. One milliliter of substrate working solutions were injected into the lower part of 120-mL oxygen flasks (final concentration, 20 µmol $L^{-1}$; oxygen, approximately 5 µmol $L^{-1}$) using disposable syringes after filling with sample water and before starting the incubation by addition of [$^{14}$C]-bicarbonate. All incubations were made in duplicate and analysis of samples was performed as described above.

Sulfide-spiking experiments were performed to estimate the relation between sulfide concentrations and $CO_2$ fixation. Increasing amounts of sodium sulfide working solution were added to water samples from the depth of maximum $CO_2$ fixation (216 m water depth), resulting in final sulfide concentrations of 10–110 µmol $L^{-1}$. Sulfide concentrations were confirmed by colorimetric analysis of control samples. Duplicate incubations were started and analyzed as described above.

### Bacterial numbers and biomass

Flowcytometric estimation of bacterial numbers was performed according to the protocol by Gasol *et al.* (2004). Briefly, water samples of 4 mL were fixed by a mixture of 1% paraformaldehyde and 0.05% glutaraldehyde (final concentration). After fixation, samples were frozen in liquid nitrogen and stored at −20 °C on board, later at −80 °C, until measurement. The thawed samples were diluted 10–20-fold by MilliQ water and stained for 15 min with Syto13 (2.5 µmol $L^{-1}$ final concentration; Molecular Probes). Samples were processed at a low speed (approximately 15 µL $min^{-1}$) in a Beckton & Dickinson FACScalibur equipped with a 488-nm laser until about 10 000 events were recorded. Fluorescent latex beads (diameter 0.92 µm; Polyscience) were used as an internal standard and for instrument adjustments. Bacteria were identified by their signature in plots of side scatter vs. green fluorescence. Cell densities were determined from the flow rate. Bacterial biomasses were calculated assuming 20 fg C per cell (Lee & Fuhrman, 1987).

## Results

### Hydrography

The Gotland Basin is characterized by a thermohaline stratification (Feistel *et al.*, 2003; Neretin *et al.*, 2003) (Fig. 1a). A thermocline at around 40 m water depth and a permanent halocline at 75 m separated the water column into three layers: the upper warmer water, the cold winter water, and the long-term stagnant deep water. A large inflow of oxygenated seawater during spring 2003 caused complete oxidation of the sulfidic bottom water (Feistel *et al.*, 2003). Since summer 2003, stagnant temperature and salinity profiles indicating the lack of significant lateral water mixing beneath 142 m water depth led to the re-establishment of sulfidic bottom waters. Because of oxygen limitation, the oxic–anoxic interface moved from the sediment surface up to 225 m in August 2004 (Labrenz *et al.*, 2005) and further to 202–206 m during the present study in May 2005.

### Chemistry

At the water surface, supersaturated oxygen concentrations of about 450 µmol $L^{-1}$ were accompanied by nitrate concentrations below the detection limit. The inventory of oxygen

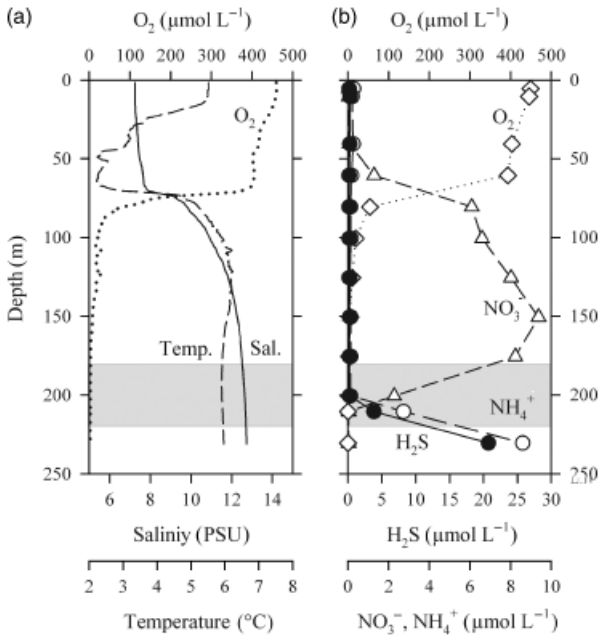


**Fig. 1.** Depth profiles of temperature, salinity, and oxygen (sensor) (a), oxygen (titrated), nitrate, ammonium, and sulfide (b) of the water column at eastern Gotland Basin (May 4, 2005). The shaded area denotes the section of the water column shown in Fig. 2.

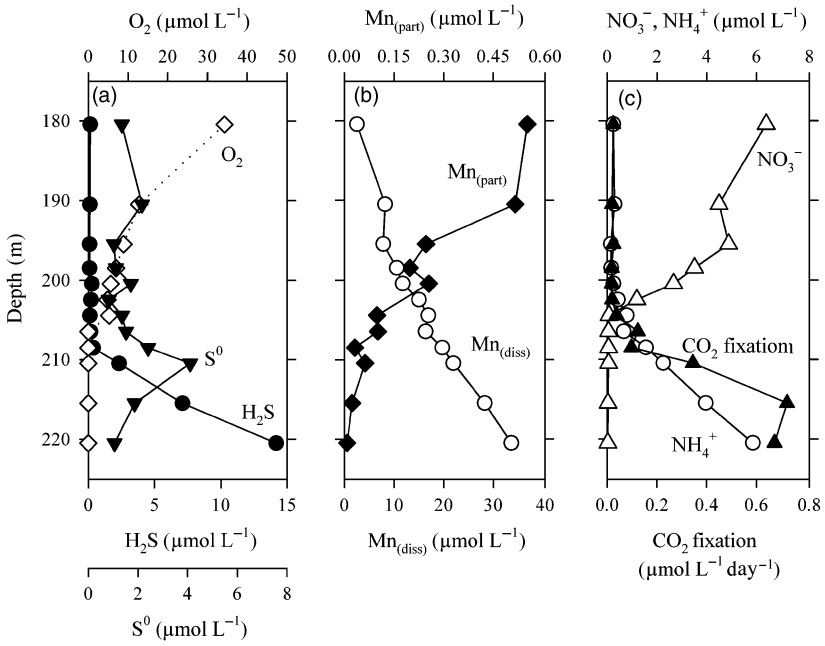


**Fig. 2.** High-resolution profiles from 180 to 220 m of oxygen, zero-valent sulfur, and sulfide (a), particulate and dissolved manganese (b), as well as nitrate, ammonium, and dark $CO_2$ fixation (c) across the redoxcline (May 4, 2005).

below the halocline was about 2 mol $m^{-2}$ and that of nitrate reached about 900 mmol $m^{-2}$. At the chemocline between 202 and 206 m water depth, oxygen, nitrite, nitrate, and sulfide were undetectable, with very little variation during the entire sampling campaign (Fig. 2a and c).

Below the chemocline, oxygen and nitrate were absent and sulfide increased steadily by about 1.1 μmol $L^{-1}$ $m^{-1}$ to approximately 20 μmol $L^{-1}$ at 226 m. The sulfide concentrations near the sediment surface varied locally from about 25 to about 50 μmol $L^{-1}$ (data not shown). Ammonium was detected 4 m above detectable sulfide and increased to about 8 μmol $L^{-1}$ at 226 m (Fig. 2a and c). Dissolved and particulate manganese was detected between 180 and 230 m. Above the sediment surface, at 230 m, the concentration was 42 μmol $L^{-1}$ (data not shown). Particulate manganese reached an inventory of nearly 10 mmol $m^{-2}$ in the water layer between 180 and 230 m, whereas the inventory of dissolved manganese in this layer exceeded 500 mmol $m^{-2}$.

Assuming a mixing coefficient for the Gotland Basin of 0.053 $cm^2$ $s^{-1}$ (Schneider *et al.*, 2002), the flux of oxygen and nitrate, and the settling of manganese particles into the oxic–anoxic interface amounted to 0.60, 0.26, and 0.025 mmol $m^{-2}$ $day^{-1}$, respectively. The calculated fluxes of sulfide, ammonium, and dissolved manganese into the oxic–anoxic interface were 0.52, 0.17, and 0.56 mmol $m^{-2}$ $day^{-1}$, respectively. Although sulfide and ammonium flux were similar to previous investigations, the dissolved manganese flux was about 10-fold higher and not balanced by the flux of manganese particles as compared with the period between 1999 and 2003, before the oxidation of the Basin (Neretin *et al.*, 2003).

The concentrations of thiosulfate and sulfite were $< 50$ nmol $L^{-1}$ throughout the entire redoxcline. Elemental sulfur varied between 0.8 and 4.2 μmol $L^{-1}$, with a maximum at 210 m (Fig. 2a), which was also confirmed by microscopic observations of filtered water samples showing a high density of yellowish sulfur globules at this depth.

### *In situ* $CO_2$ dark fixation

From 180 to 225 m, fixation of $CO_2$ was detectable (Fig. 2c). Above 205 m, the fixation rate was $< 0.1$ μmol C $L^{-1}$ $day^{-1}$. Below 205 m, it increased steadily to maximum values between 0.7 and 1.1 μmol C $L^{-1}$ $day^{-1}$ at 215 m water depth. The maximum $CO_2$ fixation was thus about 10 m below the oxic–anoxic interface within the anoxic environment. A slight decrease was observed below 215 m and an almost complete decline followed below 225 m. The integrated $CO_2$ fixation in the anoxic layer was about 9.3 mmol C $m^{-2}$ $day^{-1}$.

### Sulfide-spiking experiments

As described above, the *in situ* $CO_2$ fixation decreased substantially if sulfide concentrations exceeded 25 μmol $L^{-1}$. A comparison of $CO_2$ fixation and sulfide profiles shows that the presence of small concentrations of sulfide significantly stimulates $CO_2$ fixation in comparison with the situation just above the oxic–anoxic interface (Fig. 3). The peak of $CO_2$ fixation was always found in a water depth of around 215 m with sulfide concentrations between 5 and 10 μmol $L^{-1}$. $CO_2$ fixation decreased with further increasing sulfide concentrations below 215 m, but remained significantly higher than above the chemocline until sulfide

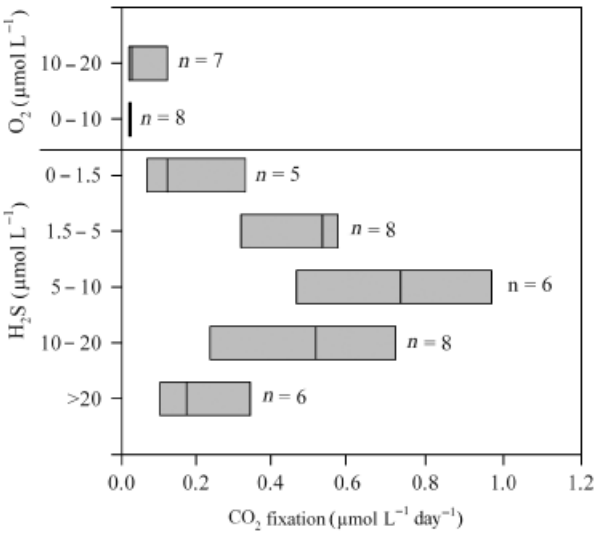


**Fig. 3.** Box plot giving the 25th, 75th percentile, and median of grouped *in situ* dark $CO_2$ fixation rates vs. *in situ* concentrations of oxygen and sulfide. The number of measurements included in each box is given.

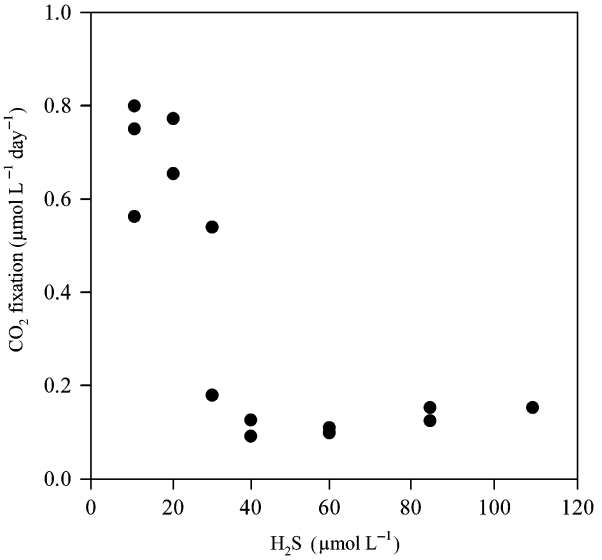


**Fig. 4.** Impact of sulfide spiking on dark $CO_2$ fixation rates at the fixation maximum (215 m). The final sulfide concentrations after spiking are given.

concentrations exceeded 20 µmol $L^{-1}$ (Figs 2c and 3). This phenomenon was further addressed by spiking samples from the fixation maximum with sodium sulfide (Fig. 4). At *in situ* sulfide concentrations of 11 µmol $L^{-1}$, the fixation was between 0.57 and 0.8 µmol C $L^{-1}$ $day^{-1}$. The addition of 10 µmol $L^{-1}$ sulfide had no effect; however, the fixation rate decreased by the addition of 20 µmol $L^{-1}$ sulfide and declined to values $< 0.2$ µmol C $L^{-1}$ $day^{-1}$ by addition of $> 20$ µmol $L^{-1}$ sulfide (Fig. 4).

### Effects of substrate spiking on *in situ* $CO_2$ dark fixation

Three distinct water layers were chosen to test the effect of substrates, which potentially fuel or inhibit autotrophic $CO_2$ fixation: (1) the layer of just elevated $CO_2$ fixation beneath the chemocline (204 m, 0.54–0.59 µmol C $L^{-1}$ $day^{-1}$, 2 µmol $L^{-1}$ sulfide); (2) the layer of maximum $CO_2$ fixation (214 m, 0.56–0.8 µmol C $L^{-1}$ $day^{-1}$, 11 µmol $L^{-1}$ sulfide); and (3) the layer with declining fixation rates beneath the fixation maximum (226 m, 0.09–0.18 µmol C $L^{-1}$ $day^{-1}$, 48 µmol $L^{-1}$ sulfide).

Near the oxic–anoxic interface (204 m), thiosulfate and polysulfide stimulated the fixation rate by 35% and 50%, respectively (Fig. 5). The addition of oxygen, nitrate, as well as manganese oxide had no effect. Dithionite reduced the fixation rate by 50%; iron oxide and sulfide reduced the rate by 75–80%. AQDS reduced the rate to $< 0.05$ µmol C $L^{-1}$ $day^{-1}$ in all three depths (Fig. 5).

None of the tested compounds significantly stimulated $CO_2$ fixation in water samples from the fixation maximum (214 m) or below (226 m). Although oxygen, thiosulfate, polysulfide, and iron oxide had neither a stimulating nor an inhibiting effect, nitrate, manganese oxide, sulfide, and dithionite significantly reduced the fixation rate (30% to $>$ 80% reduction). The addition of nitrate led to an increase of fixation by 0.2 µmol C $L^{-1}$ $day^{-1}$ in one incubation at 226 m water depth (Fig. 5).

### Total cell numbers and biomass

Total cell numbers were determined between 180 and 220 m water depth. The highest total cell densities ($2.25 \times 10^6$ cells $mL^{-1}$) were found within a 10-m layer at the oxic–anoxic interface. Above and below this layer, cell densities were between 1.3 and $1.5 \times 10^6$ cells $mL^{-1}$. Assuming an average cellular carbon content of 20 fg, the standing stock of microbial biomass ranges between 1.8 and 3.8 µmol C $L^{-1}$ (Fig. 6). At the $CO_2$ fixation maximum, up to 40% of the microbial biomass was renewed daily through inorganic carbon assimilation. Integrated over the entire redoxcline, dark $CO_2$ assimilation replaced about 18% of the bacterial biomass per day.

## Discussion

### $CO_2$ dark fixation beneath the chemocline

$CO_2$ fixation was analyzed along the oxygen and sulfide gradients. The comparison of chemical profiles and $CO_2$ fixation rates suggests that only the $CO_2$ fixation directly at the oxic–anoxic interface could be related to the activity of aerobic or denitrifying bacteria (Fig. 2a and c). Within this depth interval, the corresponding $CO_2$ fixation was about

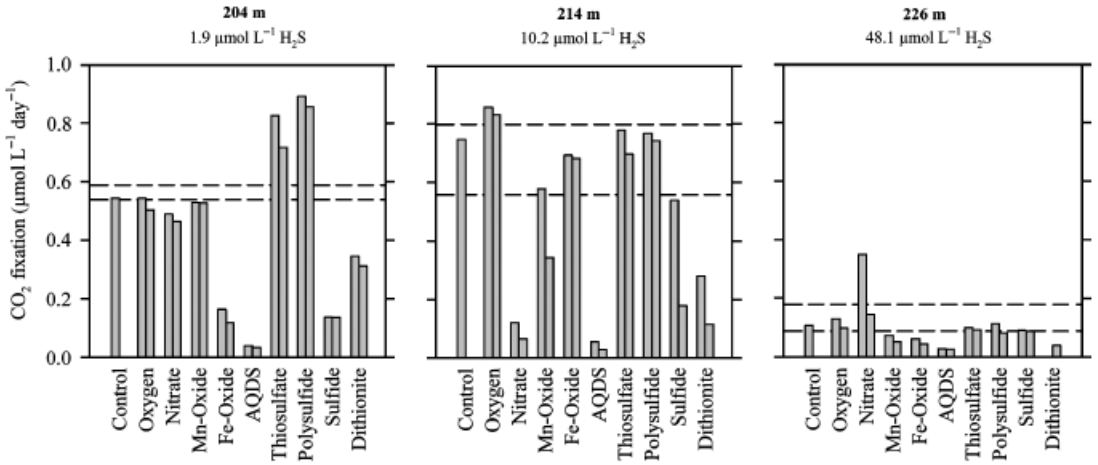


**Fig. 5.** Effect of added potential electron donors, acceptors, and reducing agents (final concentration 20 µmol L$^{-1}$, oxygen approximately 5 µmol L$^{-1}$) on the dark $CO_2$ fixation rate above (204 m), within (214 m), and beneath the fixation maximum (226 m depth) with increasing *in situ* sulfide concentrations. Dashed lines represent the two parallel determinations of the *in situ* $CO_2$ fixation.

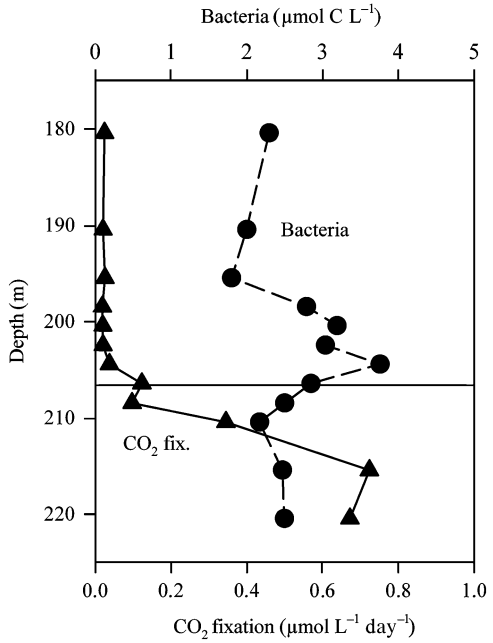


**Fig. 6.** Bacterial biomass and dark $CO_2$ fixation across the redoxcline (May 4, 2005). The vertical line indicates the depth of the chemocline.

0.03 mmol C m$^{-3}$ day$^{-1}$. Given that nitrifying bacteria oxidize about 8.3 mol ammonia mol$^{-1}$ $CO_2$ assimilated into biomass (Billen, 1976), the ammonia flux of 0.17 mmol m$^{-2}$ day$^{-1}$ would account for the assimilation of 0.01 mmol $CO_2$ m$^{-3}$ day$^{-1}$ within a 2-m layer of active ammonia-oxidizing bacteria, which would be one-third of the measured amount. In contrast, the flux of $Fe^{2+}$ is too low to account for significant $CO_2$ fixation by iron-oxidizing bacteria. The remaining $CO_2$ assimilation may therefore be due to aerobic as well as nitrate-dependent sulfur-oxidizing bacteria.

The fixation maximum was located 10 m beneath the oxic–anoxic interface and, therefore, clearly under anoxic conditions (Figs 2 and 3), and was > 20-fold higher than directly at the interface. The maximum $CO_2$ fixation rates were in the same range as reported from other pelagic oxic–anoxic interfaces (Jørgensen *et al.*, 1991; Mandernack & Tebo, 1999; Taylor *et al.*, 2001; Zopfi *et al.*, 2001) (Table 1). Significantly higher fixation rates were reported from Framvaren Fjord and Mariager Fjord, respectively, which showed much steeper gradients of sulfide (Mandernack & Tebo, 1999; Zopfi *et al.*, 2001) (Table 1).

Jørgensen *et al.* (1991) determined a molar ratio of *in situ* $CO_2$ fixation to *in situ* $^{35}$S-sulfide oxidation of 0.13–0.17 for the Black Sea chemocline, which is well in the range of sulfide-oxidizing *Thiomicrospira denitrificans* and *Thiobacillus denitrificans* strains in denitrifying chemostats (0.23 and 0.4, respectively) (Timmer-ten Hoor, 1981). However, given these molar ratios, the sulfide flux in pelagic marine chemoclines only accounts for a small percentage of the measured dark carbon fixation (Jørgensen *et al.*, 1991; Taylor *et al.*, 2001; Zopfi *et al.*, 2001). Assuming a molar ratio of 0.14 (Taylor *et al.*, 2001), the sulfide flux at the Gotland Basin chemocline would account for about 0.8% of the electron donor demand during the present study, which suggests that either the sulfide fluxes are strongly underestimated by current flux-balance models or that the sulfide flux indeed only plays a minor role in microbial chemoautotrophy.

Spiking experiments were conducted to investigate whether microbial chemoautotrophy at pelagic marine redoxclines is indeed sulfide-based and may be limited *in situ* by the flux of either electron donors or acceptors.

**Table 1.** Comparison of variables relevant to chemolithotrophic driven dark $CO_2$ fixation from different marine environments with pelagic redoxclines

| Site | Maximal rate $CO_2$ fixation ($\mu$mol C $L^{-1}$ $day^{-1}$) | Total assimilation $CO_2$ fixation (mmol C $m^{-2}$ $day^{-1}$) | Integrated depth (m) | $H_2S$ gradient ($\mu$mol $L^{-1}$ $m^{-1}$) | Reference |
|---|---|---|---|---|---|
| Black Sea | 0.19 | 2.0 | 14 | 0.68 | Jørgensen *et al*. (1991) |
| | 0.32 | 5.3 | 25 | 0.51 | Jørgensen *et al*. (1991) |
| Baltic Sea | 4.5* | 36* | 23* | 2.0* | Gocke (1989) |
| | 4.6† | 34† | 23† | 3.6† | Detmer *et al*. (1993) |
| | 1.1 | 9.3 | 21 | 1.1 | This study |
| Cariaco Basin | 0.33 | 14.2 | 136 | NR | Tuttle & Jannasch (1979) |
| | 0.4–2.5 | 26–157 | 80–100 | 0.08–0.18 | Taylor *et al*. (2001) |
| Mariager Fjord | 86 | 10.4 | 0.3 | > 65‡ | Zopfi *et al*. (2001) |
| Framvaren Fjord | 5.8/11.2 | NR | NR | > 50§ | Mandernack & Tebo (1999) |

*Estimated from Gocke (1989, fig. 2).
†Estimated from Detmer *et al*. (1993, fig. 2).
‡Estimated from Zopfi *et al*. (2001, fig. 6).
§From Millero (1991).
NR, not reported.

The results revealed a significant stimulation only by thiosulfate and polysulfide at 204 m, suggesting electron donor limitation in this layer, whereas no stimulation by sulfur compounds was observed at the fixation maximum or below (Fig. 5). Interestingly, $CO_2$ fixation appeared to be susceptible to sulfide concentrations $> 30\,\mu M$ *in situ* as well as in spiking experiments (Figs 2–5). In fact, a similar restriction of $CO_2$ fixation to low sulfide concentrations might also be present at the Black Sea redoxcline (Jørgensen *et al*., 1991), and even at the Mariager Fjord with steeper gradients (Zopfi *et al*., 2001). $CO_2$ fixation was also strongly susceptible to spiking of sodium dithionite and AQDS, whereas dithionite is unstable and rapidly decays into thiosulfate and sulfite in an aqueous solution, accompanied by strong reduction of the redox potential ($E_0' \sim -550$ mV), which could have caused a toxic effect. AQDS mediates rapid electron transfer, for example between bacteria and metal oxide surfaces and is therefore commonly used as a humic acid analogue (Benz *et al*., 1998; Newman & Kolter, 2000; Burgos *et al*., 2003; Rosso *et al*., 2004). It rapidly oxidizes free sulfide (van der Zee *et al*., 2001), but due to its lower midpoint potential ($E_0' \sim -184$ mV), it does not interact with $Fe^{2+}$ or $Mn^{2+}$ (Benz *et al*., 1998) and does not oxidize hydrogen in the absence of a palladium catalyst (e.g. Lovley *et al*., 1999). However, spiking of AQDS caused the strongest reduction of $CO_2$ fixation above, within, and below the fixation maximum (Fig. 5). Although we cannot completely exclude that even these extremely low AQDS concentrations may be toxic to some microorganisms, these findings suggest that sulfide is indeed involved in the *in situ* metabolism of chemolithoautotrophs and that elevated sulfide concentrations itself, significant changes of the redox potential, or a combination may exert toxic effects on autotrophic microorganisms beneath pelagic chemoclines.

Surprisingly, none of the tested electron-accepting compounds significantly stimulated $CO_2$ fixation (Fig. 5). Above the fixation maximum, chemolithoautotrophic microorganisms were probably sulfur-limited. Therefore, electron acceptors could have no effect on $CO_2$ fixation. Within the fixation maximum, no stimulation of autotrophic activity was expected, as electron-donating and -accepting compounds should be available in balanced amounts. The addition of single electron-donating or -accepting compounds could thus not lead to increased $CO_2$ assimilation. Beneath the fixation maximum, a possible electron acceptor limitation was expected, but none of the tested electron acceptors stimulated $CO_2$ assimilation. The absence of oxygen and nitrate in this layer may explain why these electron acceptors did not instantaneously stimulate $CO_2$ fixation, whereas such stimulation has previously been observed in long-term enrichment experiments (Labrenz *et al*., 2005), where significant microbial growth is possible. Despite the detection of particulate manganese in anoxic water, manganese oxide amendments did not stimulate $CO_2$ fixation. Manganese particles from the chemocline, analyzed by fluorescence microscopy, were highly colonized by microorganisms (data not shown). The addition of sterile manganese oxide particles may not provide a significant amount of electron acceptors unless bacteria have colonized these particles. However, stimulation by oxidized manganese and iron was observed by Taylor *et al*. (2001) in deeper anoxic layers of the Cariaco Basin.

## Chemical vs. biological oxidation of sulfide in the presence of manganese oxide

The intensive manganese cycle and the presence of particulate manganese in sulfidic water are likely responsible for the

lacking overlap of sulfide and nitrate found during the present study. Manganese particles settle down much faster than diffusion of oxygen and nitrate proceeds (Neretin *et al.*, 2003), and thus may become available for sulfide oxidation deeper in the water column and hence deplete sulfide before nitrate and oxygen become available. Similar observations have been made in the Cariaco Basin, underlining the possible importance of manganese oxide as an electron acceptor (Ho *et al.*, 2004).

The chemical profiles and the very low concentrations of thiosulfate and sulfite suggest a strong biological control of sulfide oxidation beneath the Gotland Basin chemocline in the presence of manganese oxides. However, several studies have provided strong evidence that chemical oxidation of free sulfide, iron sulfide, and pyrite by manganese oxide is fast, if not even spontaneous (Burdige & Nealson, 1986; Yao & Millero, 1993; Schippers & Jørgensen, 2001). Yao & Millero (1993) determined the kinetics of sulfide oxidation ($-\mathrm{d}[H_2S]_T/dt$) by manganese oxide in seawater to be first order with respect to total sulfide ($[H_2S]_T$) and manganese oxide concentrations ($[MnO_2]$):

$$-\mathrm{d}[H_2S]_T/\mathrm{d}t = k \times [H_2S]_T \times [MnO_2] \quad (1)$$

Throughout the oxic–anoxic interface of the Gotland Basin, sulfide concentrations of up to $10\,\mu mol\,L^{-1}$ and manganese oxide concentrations of $0.1–0.02\,\mu mol\,L^{-1}$ at $6\,^{\circ}C$ ($k = 293\,M^{-1}\,min^{-1}$) would result in a chemical manganese-dependent sulfide oxidation of $0.05–0.07\,\mu mol\,L^{-1}\,day^{-1}$, i.e. a daily oxidation of $< 1\%$ of the present sulfide. Thus, in contrast to marine sediments, sulfur-oxidizing microorganisms could successfully compete with chemical sulfide oxidation in the presence of manganese oxide at pelagic oxic–anoxic interfaces.

### Autotrophic microbial sulfur metabolism in the absence of oxygen and nitrate

In the absence of oxygen and nitrate, microorganisms could catalyze the autotrophic disproportionation of sulfur species (Bak & Cypionka, 1987). However, under *in situ* conditions at the fixation maximum, the chemical oxidation of sulfide by manganese oxide is far too slow to supply sufficient sulfur intermediates for the energetically unfavorable sulfur disproportionation to account for the detected *in situ* $CO_2$ fixation. Jørgensen & Bak (1991) and Ho *et al.* (2004) argued that manganese oxide or iron oxide could serve as the terminal electron acceptors for sulfur-oxidizing bacteria under such conditions. This view is supported by the detection of particulate manganese down to the $CO_2$ fixation maximum at the eastern Gotland Basin (Fig. 2b), the observed susceptibility to high sulfide concentrations, and reduction of the redox potential (Fig. 4), as well as the dense colonization of manganese-rich particles. However, to date, there exists no description of a pure culture yielding such a reaction.

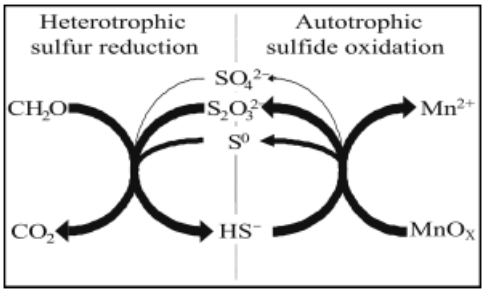


**Fig. 7.** Proposed scheme of the interaction between incomplete sulfide oxidation coupled to electron acceptor reduction (e.g. $MnO_x$) and heterotrophic sulfur reduction at the dark $CO_2$ fixation maximum beneath the chemocline of the eastern Gotland Basin.

### Sulfide recycling by heterotrophic microorganisms

Beneath the Black Sea chemocline, sulfide was oxidized to sulfur intermediates dominated for up to 80% by thiosulfate (Jørgensen *et al.*, 1991; Elsgaard & Jørgensen, 1992). This thiosulfate is then recycled to sulfide by heterotrophic microorganisms. Such organisms have been identified by molecular surveys (Madrid *et al.*, 2001; Vetriani *et al.*, 2003; Labrenz *et al.*, 2005), and have also been repeatedly isolated from the chemocline of several anoxic basins (Tuttle & Jannasch, 1973; Nealson *et al.*, 1991; Brettar *et al.*, 2002; Nealson & Scott, 2006). Although sulfate reduction rates were extremely low beneath the chemocline of the Gotland Basin, and the Black Sea, respectively (L. Neretin, pers. commun.; Jørgensen *et al.*, 1991), a recycling of $43–65\%\,day^{-1}$ of the standing stock of sulfide by heterotrophic sulfur reducers would be sufficient to support the detected $CO_2$ fixation (Fig. 7). The importance of thiosulfate as an intermediate in the sulfur cycle is well recognized for marine sediments (Jørgensen, 1990), but it seems likely that the same is also true for pelagic chemoclines.

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## Authors' contribution

G.J. and W.M.-H. contributed equally to this work.

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