Sulfide Oxidation and Speciation of Sulfur Intermediates in Marine Environments

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Preface

This Ph.D. thesis deals with biogeochemical aspects of the oxidative part of the sulfur cycle. One part of the project was a comparative study on the chemical speciation and distribution of partially oxidized sulfur compounds in different environments. In addition, sulfide oxidation was more deeply investigated in the chemocline of a stratified fjord and *Thioploca*-inhabited surface sediments from the upwelling area off Central Chile. The financial support for this thesis came from the Max Planck Society and the German Ministry of Education and Research (BMBF) and is gratefully acknowledged.

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CHAPTER 1

1. Introduction

1.1 Organic Matter Degradation and Sulfate Reduction

All biogeochemical cycles of the major elements (C, H, N, O, S, P, Fe, Mn) in the marine environment are ultimately linked and driven by energy captured by phototrophic organisms. Algae but also cyanobacteria use light to split water into oxygen, protons and electrons which are used for the reduction of CO_2, the assimilation of nutrients and the build up of biomass. The energy stored in the biomass is released and supports the life of aerobic heterotrophic organisms as the dead algae are degraded while sinking down the water column. The aerobic mineralization of organic matter (1) is very efficient and thus only a fraction of the originally formed biomass is deposited on the seafloor. Whereas in shelf areas about 10 to 50% of the primary production reach the sediment surface, the value decreases to 1% or less for deep-sea sediments (Jørgensen, 1983; Suess, 1980). Due to the higher input and better degradability of organic matter to sediments of coastal margins the mineralization rates are generally higher and, as a consequence, oxygen penetration is only a few millimeter compared to several centimeter in deep-sea sediments. Below the oxic surface layer the organic matter is degraded anaerobically by a diverse community of bacteria with different physiological capabilities. In the first step, fermenting bacteria hydrolyze large organic compounds, e.g. proteins and polysaccharide and produce small molecules like short chain fatty acids and H_2 which can be used by anaerobically respiring bacteria in the second step. Instead of oxygen they use one or more alternative electron acceptors for the oxidation of organic matter (2-5). In ideal cases a sequential use of these alternative electron acceptors is observed where first NO_3^-, then Mn(IV)oxides, Fe(III)oxides, SO_4^{2-} and finally CO_2 is reduced (Froelich et al., 1979).

$$CH_2O + O_2 + OH^- \Rightarrow HCO_3^- + H_2O \quad (1)$$

$$5CH_2O + 4NO_3^- \Rightarrow 5\,HCO_3^- + 2\,N_2 + 2\,H_2O + H^+ \quad (2)$$

$$CH_2O + 2MnO_2 + H^+ \Rightarrow HCO_3^- + 2\,Mn^{2+} + H_2O + OH^- \quad (3)$$

$$CH_2O + 4FeOOH \Rightarrow HCO_3^- + 4\,Fe^{2+} + 6\,OH^- \quad (4)$$

$$\mathbf{2CH_2O + SO_4^{2-} + H^+ \Rightarrow 2\,HCO_3^- + HS^-} \quad (5)$$

$$2CH_2O + OH^- \Rightarrow HCO_3^- + CH_4 \quad (6)$$

The relative importance of the different mineralization pathways in marine sediments has been extensively studied in recent years (Canfield et al., 1993; Thamdrup and Canfield, 1996). In most marine sediments denitrification (2) seems to be a minor pathway for organic matter oxidation. However, it may become much more important in sediments covered with oxygen

depleted, but nitrate rich bottom water (discussed in Canfield, 1993). Only little is known about the quantitative contribution of Mn(IV) and Fe(III) reduction to carbon oxidation (3, 4). However, the few available reports suggest that dissimilatory metal reduction is much more important than previously thought, although it may also vary considerably between different sites. For instance, in manganese rich slope sediments from the Skagerrak Mn(IV) reduction was responsible for about 90% of organic carbon mineralization, but was completely absent in manganese poor sediments from a shallower station (Canfield, 1993). Depending on the type of sediment, dissimilatory Fe(III) reduction was responsible for up to 50% of organic matter oxidation (Canfield, 1993). As it has been shown in the same and other studies (e.g. Jørgensen, 1982; Canfield, 1991) sulfate reduction becomes increasingly important at shallower sites with a higher input of organic matter and hence increased mineralization rates. In coastal sediments sulfate reduction becomes the dominant anaerobic degradation process and is typically responsible for about 50% of the total carbon mineralization. This portion may almost reach 100% in upwelling areas (Thamdrup and Canfield, 1996) and euxinic environments. Together with the estimates of about 90% of the global primary production being buried and degraded in shelf sediments (Berner, 1989; Hedges and Keil, 1995), makes dissimilatory sulfate reduction the second most important mineralization process, after aerobic respiration. Methanogenesis is the last step in the anaerobic chain of organic matter degradation (6, simplified). Methane is mainly formed by cleavage of acetate or by the reduction of CO_2 with H_2. Since both, H_2 and acetate are preferred substrates of sulfate reducing bacteria and since they have a higher affinity towards these compounds, methane formation is inhibited as long as sulfate is present in the porewater. Below the sulfate reduction zone methane formation can proceed down to several meters depth.

1.2 Oxidative part of the sulfur cycle

Sulfate reduction as measured using $^{35}SO_4^{2-}$, is one of the few mineralization processes, which can be quantified easily. From the amount of reduced ^{35}S that is produced over time, the volumetric sulfate reduction rate can be calculated. Mass balance calculations show that only about 10% of the sulfide which is produced via sulfate reduction is ultimately buried in the sediment, mostly in form of pyrite (Jørgensen, 1977). The remaining portion is reoxdized to sulfate by various competing chemical and biological processes.

Oxic sulfide oxidation

Under certain sedimentary conditions, dissolved sulfide diffuses to the surface where it is chemically oxidized by dissolved oxygen according to the overall reaction (7). Where the supply of oxygen is limited, e.g. in fjords and deep basins, sulfide may even escape into the water column where it is oxidized in the redox transition zone (chemocline).

$$HS^- + 2O_2 \Rightarrow SO_4^{2-} + H^+ \quad (7)$$

However the chemistry of the reaction is not as simple as the stoichiometry implies, and the exact reaction mechanism still remains to be elucidated (Zhang and Millero, 1993). A number of studies have shown, that the oxidation of sulfide does not directly lead to sulfate but passes through several intermediates of different oxidation states (e.g. Avrahami and Golding, 1968; Cline and Richards, 1969; Chen and Morris, 1972; Zhang and Millero, 1993). Among them, sulfite is usually the first product formed (8)

$$HS^- + 1.5O_2 \Rightarrow HSO_3^- \quad (8)$$

The rapid oxidation of sulfite with oxygen explains the sulfate formation, which is commonly observed during sulfide oxidation experiments (9). Sulfite can also react with HS^- to form thiosulfate ($S_2O_3^{2-}$) (10).

$$SO_3^{2-} + 0.5O_2 \Rightarrow SO_4^{2-} \quad (9)$$

$$SO_3^{2-} + 0.5O_2 \Rightarrow S_2O_3^{2-} + OH^- \quad (10)$$

In most of the chemical studies thiosulfate and sulfate were the only stable oxidation products and accumulated during the course of the experiments.

In presence of trace metals, as typical for natural environments, the formation of elemental sulfur in the initial step is also possible (11) (Steudel, 1996, Zhang and Millero, 1993).

$$2HS^- + O_2 \Rightarrow 2S^0 + 2OH^- \quad (11)$$

Elemental sulfur can react with sulfite and sulfide to form thiosulfate (12) and polysulfides (13), respectively.

$$S^0 + SO_3^{2-} \Rightarrow S_2O_3^{2-} \quad (12)$$

$$(n-1)S^0 + HS^- \Rightarrow HS_n^- \quad (13)$$

However, polysulfides are not stable under oxic condition and rapidly decompose to thiosulfate and elemental sulfur (Steudel et al., 1986). Thus, based on these summarized results from chemical studies, only elemental sulfur and thiosulfate may be expected to be present under oxic conditions.

Although sulfide is basically a waste product of sulfate reducing bacteria, it still contains a considerable amount of the energy originally stored in the biomass of primary producers.

Aerobic lithotrophic bacteria can thrive on the oxidation of sulfide or sulfur intermediates with oxygen. Because chemical sulfide oxidation can be very fast in the environment, bacteria had to develop strategies to successfully compete for sulfide. The most important adaptations are high enzyme affinities towards O_2 and sulfide, and motility. Motility enables the organisms to position themselves in the oxic/anoxic interface where both, oxygen and sulfide, are present in low concentrations and are only supplied by diffusion (Nelson et al., 1986). Under such low reactant conditions chemical sulfide oxidation becomes much slower due to the second order kinetics of the reaction (Zhang and Millero, 1994). Because of the very low saturation constants for oxygen and sulfide of 1 μM or below in chemolithotrophic sulfur bacteria (Kuenen and Bos, 1989; van den Ende and van Gemerden, 1993), these organisms can still metabolize at maximal rates and may outcompete the non-biological sulfide oxidation. The main product of biological sulfide oxidation is sulfate. Sulfur intermediates are only formed transiently under changing environmental conditions and severe oxygen limitation (van den Ende and van Gemerden, 1993).

Anoxic sulfide oxidation

In most marine sediments sulfide does not diffuse to the sediment surface, but is removed from the porewater below the oxidized surface layer, in the suboxic zone, by oxidation and precipitation. This zone is characterized by the absence of oxygen and sulfide, but increased concentrations of dissolved reduced iron and manganese.

For the chemical oxidation of sulfide in marine sediments only manganese(IV)oxides (14) and iron(III)oxides (15) are of importance since the reaction with nitrate is kinetically unfavorable. Similar to the oxic pathways of sulfide oxidation, sulfur intermediates are also formed during anoxic oxidation of sulfide.

$$\delta MnO_2 + HS^- + 3H^+ \Rightarrow Mn^{2+} + S^0 + 2H_2O \qquad (14)$$

For instance, elemental sulfur is a main product of the sulfide oxidation with Mn(IV) (Burdige and Nealson, 1986), but with increasing MnO_2/H_2S-ratios thiosulfate and especially sulfate become more important as products (Yao and Millero, 1993). The stoichiometry in E.g. 14 is thus an oversimplification and describes only approximately the situation for a 1:1 ratio between sulfide and manganese.

In most marine sediments iron is much more abundant than manganese and is responsible for the efficient removal of dissolved sulfide from the interstitial water (Canfield, 1989). Unlike manganese, iron(III)oxide is a rather poor oxidant for the complete oxidation of sulfide to sulfate (Aller and Rude, 1988; King, 1990; Elsgaard, 1992). During the reaction of sulfide with iron(III)oxides, dissolved ferrous iron and elemental sulfur are produced (15).

$$2FeOOH + HS^- + 6H^+ \Rightarrow 2Fe^{2+} + S^0 + 4H_2O \quad (15)$$

Furthermore, if sulfide is present in excess, dissolved ferrous iron will be precipitated as FeS. However, the formation of thiosulfate and sulfite has also been reported (Pyzik and Sommer, 1981; dos Santos and Stumm, 1992).

The sulfur intermediates, which are formed during sulfide oxidation may be further transformed by microorganisms. In presence of a substrate all of the sulfur intermediates can be reduced back to sulfide by sulfate reducing bacteria and others (e.g. Shewanella sp.). Sulfur intermediates are also further oxidized to sulfate when a suitable electron acceptor becomes available. Under anoxic conditions nitrate and manganese(IV)oxides have been shown to be used by microorganisms as electron acceptor for complete sulfide oxidation (Elsgaard and Jørgensen, 1992; Lovley and Phillips, 1994).

The third type of metabolism responsible for the anaerobic transformation of sulfur intermediates is the so-called disproportionation (e.g. Bak and Cypionka, 1987; Thamdrup et al., 1993), which is described as a type of inorganic fermentation, where the substrate serves as electron donor as well as electron acceptor.

$$4SO_3^{2-} + H^+ \Rightarrow 3SO_4^{2-} + HS^- \quad (16)$$

$$S_2O_3^{2-} + H_2O \Rightarrow SO_4^{2-} + HS^- + H^+ \quad (17)$$

$$4S^0 + 4H_2O \Rightarrow SO_4^{2-} + 3HS^- + 5H^+ \quad (18)$$

By using radiotracers, it was shown that the disproportionation of thiosulfate is a key process in the sedimentary sulfur cycle (Jørgensen, 1990).

1.3 Sulfur intermediates in the environment

As apparent from the previous sections, sulfur intermediates are formed via nearly all-possible sulfide oxidation pathways. They have been shown to influence trace metal solubility and mobility by complexation with polysulfides and thiosulfate (Jacobs and Emerson, 1982; Morse et al., 1987). Polysulfides are suspected to be involved in the formation of pyrite (Luther III, 1991), thiols (Vairavamurthy and Mopper, 1989) and organic polysulfides (Kohnen et al., 1989). Sulfonates have been proposed to be formed by the reaction of sulfite or thiosulfate with reactive organic matter (Vairavamurthy et al., 1994).

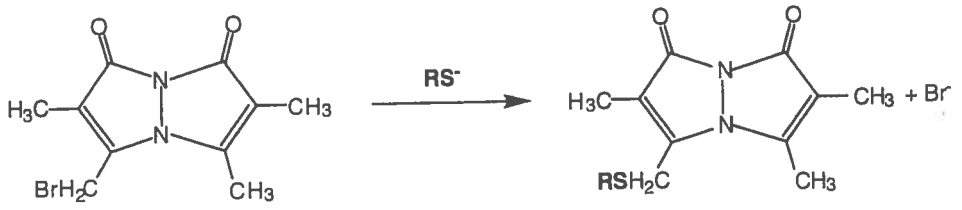
Despite the importance of sulfur intermediates for the biogeochemical cycling of carbon, manganese, iron and trace metals, comparatively little is known about their occurrence in nature. Table 1 summarizes the available data for thiosulfate and sulfite measurements in marine

environments, and illustrates the striking variability in the measured concentrations, ranging from low nM to mM. Since a wide range of different methods was used for quantification, it is unclear to what extent the variability in the data is due to the environmental conditions or the method applied. One task of this thesis was therefore to measure sulfur intermediates in different marine environments with a single method and to compare the results with previous reports.

Table 1: Concentration ranges of $S_2O_3^{2-}$ and SO_3^{2-} measurements in different marine environments. Adapted and extended from Thamdrup et al. (1994).

Ecosystem	Concentration (µM)		Method	Reference
	$S_2O_3^{2-}$	SO_3^{2-}		
Spartina -Salt Marshes				
Great Marsh, Del.	130 - 530	<0.1 - 177	Hg^{2+}-titration	Boulègue et al., 1982
Sippewissett, Mass.	<15 - 340	n.a.	Cyanolysis	Howarth et al., 1983
Sippewissett, Mass.; Great Marsh, Del.	<0.2 - 1000	0 - 7.3	Voltametry	Luther et al., 1985, 1986, 1991; Luther and Church, 1988
Mission Bay, Cal.	0 - 45	0 - 6	Bimane HPLC	Vetter et al., 1989
Unvegetated Marshes				
Mission Bay, Cal.	2 - 84	3 - 56	Bimane HPLC	Vetter et al., 1989
New York, NY	3 - 50	n.a.	Cyanolysis	Swider and Mackin, 1989
Skallingen, DK	<0.05 - 0.6	0.1 - 1.1	DTNP HPLC	Thamdrup et al., 1994
Sulfureta				
Orkneys, UK	0 - 2500	n.a.	Cyanolysis	van Gemerden et al., 1989
Texel, NL	0-35	n.a.	Cyanolysis	Visscher et al. 1992
Marine Sediments				
Walvis Bay, Namibia	15 - 145	n.a.	Hg^{2+}-titration	Boulégue and Denis, 1982
Kysing Fjord, DK	<1 - 10	n.a.	Cyanolysis	Troelsen and Jørgensen, 1982
Gulf of California	0-500	n.a.	?	Lein, 1984
Orleans, Mass.	400 - 1300	6.8 - 7.9	Voltametry	Luther et al., 1985
Aarhus; Skagerrak, DK	<0.05 - 0.45	0.1 - 0.8	DTNP HPLC	Thamdrup et al., 1994
Chesapeake Bay, Md.	0.1 - 7.1	n.a.	IP RP-HPLC	MacCrehan and Shea, 1995
Saguenay Fjord, CAN	0 - 15		Hg^{2+}-titration	Gagnon et al., 1996
Venice Lagoon, IT	0-35	n.a.	Voltametry	Bertolin et al., 1997

For this purpose, a recently developed HPLC method was chosen, where ΣH_2S, $S_2O_3^{2-}$, SO_3^{2-} and thiols in a sample are fixed by derivatization with (monobromo-)bimane (16) and subsequently analyzed by means of reversed-phase HPLC and fluorescence detection (Fahey and Newton, 1987; Rethmeier et al., 1997).



(16)

This method combines several advantages that are particularly important for environmental studies. 1. Several sulfur compounds can be measured with the same method. 2. The method is very sensitive and allows the quantification of sulfur compounds in submicromolar concentrations. 3. The sample is fixed by the derivatization step and, when kept frozen at -80, may be analyzed at a later stage. The stability of the derivate depends on the storage temperature, and the sulfur compound derivatized.

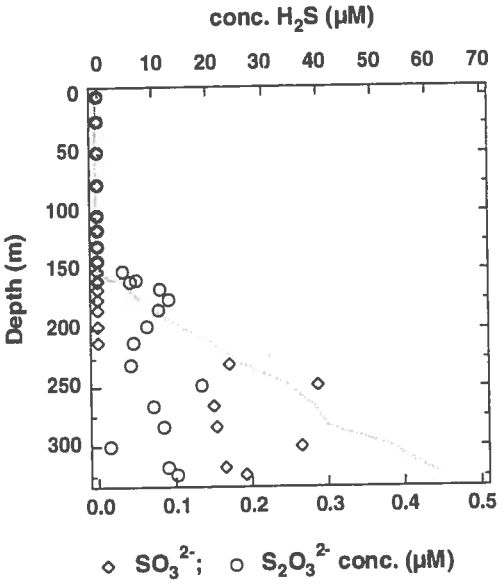


Figure 1: Distribution of thiosulfate and sulfite in the chemocline of the Black Sea (Zopfi, unpubl.). Grey line: H_2S.

To illustrate the applicability and limits of the method data from the Black Sea chemocline are presented. Figure 1 shows the first successful measurement of thiosulfate and sulfite in the chemocline of the Black Sea and illustrates the high sensitivity of the method.

Whereas sulfite is absent above and within the chemocline thiosulfate reaches a local maximum of about 100 nM in the lower chemocline at 120 m depth. Figure 1 also shows that with increasing background concentrations of ΣH_2S also the concentrations of sulfite and thiosulfate increase. Although more oxidized, sulfite reaches higher concentrations than thiosulfate below the chemocline. This observation, together with the increased scatter of the data below the chemocline, and the fact that sulfite is the first product of the reaction of sulfide with O_2, may indicate that sulfite is formed *during* the derivatization reaction. However this effect was only observed when the measurements were done at a very high sensitivity.

1.3 Aim and Outline of the Thesis

Two major goals determined the direction of this Ph.D. thesis.

1.) Reliable measurements of sulfur intermediates in nature are rare and it is not clear whether the large variability of the reported concentrations are real or due to the different methods applied. In addition, the studies often lack the spatial resolution required for a meaningful interpretation of the data. For this reason, a method for the determination of sulfur intermediates, namely sulfite and thiosulfate, was evaluated and applied to different environments. The aim of this survey was to compare the results with earlier reports and to extend the existing small database. Accurate measurements of sulfur intermediates with a high spatial resolution were expected to lead to a better understanding of sulfide oxidation in the environment. They also allow better estimates of the turnover times of thiosulfate and sulfite, and may be useful for attempts to model sulfur cycling in chemoclines and sediments.

Parts of the data, which have not been incorporated into publishable manuscripts yet, have already been presented in Chapter 1.3. A second part is included in two manuscripts (Chapter 2 and 5) and a summary of all measurements is presented in a table in Chapter 6.1.

2.) In contrast to the bacterial sulfate reduction where comparatively much is known about process rates, regulating factors and the involved microorganisms, the lack of knowledge concerning the reoxidation of sulfide is acute. The key question, of whether and to what extent sulfide is oxidized by biological or chemical processes has not been answered for most environments.

To explore the controls on the mode of sulfide oxidation and the formation and distribution of sulfur intermediates two of the surveyed environments were chosen and studied in greater detail. The first environment was the stratified water column of a brackish fjord in Denmark. This work resulted in the manuscript with the title: *"Influence of water column dynamics on sulfide oxidation and other major biogeochemical processes in the chemocline of Mariager Fjord"*, and is presented in Chapter 2.

Surface sediments from the upwelling area off Central Chile were the second investigated environments. Despite high sulfate reduction rates the concentrations of porewater sulfide are very low and sulfate is barely depleted, suggesting that sulfide is efficiently re-oxidized. Dense mats of filamentous, nitrate storing sulfur bacteria of the genera *Thioploca* and *Beggiatoa* cover the sediment during part of the year and it has been argued that they have a great influence on the sedimentary nitrogen and sulfur cycles (Fossing et al., 1995). Since nothing was known about the metabolic capabilities of *Thioploca* spp. incubation experiments with purified samples were set up to determine their physiological traits. The results of these experiments are described in the manuscript: "*Nitrogen, carbon and sulfur metabolism in natural Thioploca samples*" in Chapter 3.

More ecological aspects are described in Chapter 4 ("*Ecology of Thioploca spp.: NO_3^- and S^0 storage in relation to chemical microgradients and influence on the sedimentary nitrogen cycle*") where the influence of *Thioploca* on the sedimentary nitrate uptake was studied by NO_3^--microsensors. In addition, the hypothesis of its vertical shuttling between the sediment surface and deeper sediment layers, as it was proposed by Fossing et al. (1995), was tested.

Chapter 5 deals with the geochemical aspects of sulfide oxidation in the upwelling influenced sediments off Central Chile. The manuscript with the title "*Early diagenesis and isotope biogeochemistry of sulfur in Thioploca-dominate sediments off Chile*" contains a description of the Fe-Mn-S geochemistry at 4 different stations and a discussion on the distribution and formation of sulfur intermediates. Intensive sulfur cycling has been proposed to determine the stable isotopic composition of sedimentary sulfides (Canfield and Teske, 1996) this model was tested in these highly active surface sediments where sulfide is rapidly reoxidized.

The main results and conclusions of the manuscript presented in the previous sections are summarized in Chapter 6.

CHAPTER 2

Influence of Water Column Dynamics on Sulfide Oxidation and other Major Biogeochemical Processes in the Chemocline of Mariager Fjord (Denmark)

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Abstract

Major electron donors (H_2S, NH_4^+, Mn^{2+}, Fe^{2+}) and acceptors (O_2, NO_3^-, Mn(IV), Fe(III)), process rates ($^{35}SO_4^{2-}$ reduction, dark $^{14}CO_2$ fixation) and vertical fluxes were investigated to quantify the dominant biogeochemical processes at the chemocline of a shallow brackish fjord. Under steady state conditions, the upward fluxes of reductants and downward fluxes of oxidants in the water column were balanced. However, changes in the hydrographical conditions caused a transient non steady state at the chemocline and had a great impact on process rates and the distribution of chemical species. Maxima of S^0 (17.8 µM), thiosulfate (5.2 µM) and sulfite (1.1 µM) occured at the chemocline, but were hardly detectable in the sulfidic deep water. The distribution of S^0 suggested that the high concentration of S^0 was (I) more likely due to a low turnover than a high formation rate and (II) was only transient, caused by chemocline perturbations. Kinetic calculations of chemical sulfide oxidation based on actual conditions in the chemocline revealed that, under steady state conditions with a narrow chemocline and low reactant concentrations, biological sulfide oxidation may account for more than 88% of the total sulfide oxidation. Under non steady state conditions, where oxic and sulfidic water masses were recently mixed, resulting in an expanded chemocline, the proportion of chemical sulfide oxidation increased. The sulfide oxidation rate determined by incubation experiments was 0.216 µmol l^{-1} min^{-1}, one of the highest reported for stratified basins and about 15 times faster than the initial rate for chemical oxidation. The conclusion of primarily biological sulfide oxidation was consistent with the observation of high rates of dark $^{14}CO_2$ fixation (10.4 mmol m^{-2} d^{-1}) in the lower part of the chemocline. However, rates of dark $^{14}CO_2$ fixation were too high to be explained only by lithoautotrophic processes. CO_2 fixation by growing populations of heterotrophic microorganisms may have additionally contributed to the observed rates.

1. Introduction

Stratified basins are characterized by a density gradient in the water column that limits circulation and consequently the transport of oxidants down to the sediment. In extreme cases, the limited supply of oxygen leads to the development of anoxic conditions within the water column. Organic matter is then mainly degraded by anaerobic sulfate reducing bacteria, which produce hydrogen sulfide (H_2S). At the oxic/anoxic interface (chemocline) H_2S is oxidized, but, in contrast to bacterial sulfate reduction, H_2S oxidation proceeds through a complex web of different pathways and cycles where chemical and biological processes are working concurrently.

For decades, chemoclines in stratified water columns such as the Black Sea, Cariaco Basin or Framvaren Fjord have attracted scientists from different fields to study sulfide oxidation. Studies on the chemistry of H_2S oxidation in the laboratory (Chen and Morris, 1972; Zhang and Millero, 1993b) or in the environment (Millero, 1991a, b; Sorokin, 1970) showed sulfate (SO_4^{2-}), thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}) to be the major oxidation products. Under certain conditions, such as high sulfide to oxygen ratios, polysulfides (S_n^{2-}) and elemental sulfur (S^0) also are produced (Chen and Morris, 1972). However, little is known about regulatory factors of H_2S oxidation and the speciation and distribution of sulfur intermediates in nature. Because the concentrations of these compounds are usually very low and sensitive analytical methods have been lacking, reliable measurements of $S_2O_3^{2-}$ and SO_3^{2-} are scarce.

In environments, where the oxic/anoxic interface is shallow and the light intensity is sufficiently high, dense populations of anoxygenic phototrophic bacteria are often observed (Overmann and Beatty, 1996; Sorokin, 1970) and the contribution of these organisms to H_2S oxidation is evident. The situation is less clear in deep chemoclines below the photic zone. Although chemolithothrophic sulfur oxidizing bacteria have been frequently enriched and isolated from the oxic/anoxic interface (Jannasch et al., 1991) this approach does not give quantitative information about bacterial sulfide oxidation. In some studies it has been argued that the first step in H_2S oxidation and the production of intermediates is a chemical process (Sorokin, 1970), whereas the second step, the oxidation of intermediates to sulfate is catalyzed by bacteria. The reason for this is not clear since the kinetic rate law for H_2S oxidation predicts low oxidation rates at low reactant concentrations (Zhang and Millero, 1993b). On the other hand, chemostat studies have shown that bacteria very rapidly oxidize H_2S even at dissolved oxygen concentrations of less than 1 µM (van den Ende and van Gemerden, 1993).

The primary goal of this study was to investigate the mode (biological / chemical) of H_2S oxidation and the formation and distribution of sulfur intermediates in the chemocline of Mariager Fjord. However, during the study we observed major changes in the physical and chemical conditions in the water column. This observation allowed us to study the regulatory influence of water column dynamics on sulfide oxidation and biogeochemical processes in general, which unfortunately has been often neglected in earlier studies.

Our work was carried out in the central basin of Mariager Fjord, a long brackish fjord in North-eastern Jutland (Denmark). The central basin (27 m deep) with its stratified water column is connected to the Kattegat by a shallow (4 - 10 m deep) channel of about 20 km length. The water exchange with the sea is limited although strong winds can cause increased influx of seawater (Fenchel et al., 1995). Once every few years a complete mixing of the deep central basin is observed and low concentrations of dissolved oxygen can be found down to the bottom (Andersen et al., 1998). Such an event was reported last time in 1997 (H. Fossing, personal communication).

In this paper we present data on the dynamics of the water column stratification and its effect on biogeochemical processes in the chemocline. Rates of sulfide oxidation were determined by incubation experiments and a recently developed RP-HPLC method was applied to determine sulfur intermediates. We present estimates of the relative contribution of chemical and biological processes to sulfide oxidation. The relationship between autotrophic sulfide and ammonium oxidation and dark CO_2 incorporation in the chemocline is discussed, and budgets for organic carbon and sulfur are presented. We also identified and quantified different groups of bacteria involved in the sulfur cycle.

2. Material and Methods

2.1 Sampling

Water samples were taken in the central basin of Mariager Fjord (56°39'76"N, 09°58'50"E) on 13-16 September 1996 from RV *"Victor Hensen"* (Fig. 1). Water was continuously collected on deck, using a peristaltic pump (Heidolph, Germany) and gastight Tygon®-tubing (7 mm i.d.; Norton Plastics, Akron, Ohio). The inlet of the tubing was connected to a CTD-sensor (Meerestechnik-Elektronik GmbH, Trappenkamp, Germany) which monitored pressure, conductivity, salinity and oxygen. The sea was extremely calm, and the vertical sampling accuracy was better than ±10 cm.

2.2 Chemical measurements

Oxygen concentrations were determined by Winkler titration in 100 ml glass bottles (Grasshoff, 1983). When necessary, the interference from sulfide was eliminated by the procedure of Ingvorsen and Jørgensen (1979). Sulfide was measured colorimetrically by the methylene blue technique (Cline, 1969). Detection limits for both methods were about 1 µM.

Sulfur speciation: Water samples for thiosulfate and sulfite determinations were taken in 50 ml glass bottles. Each bottle was flushed with at least 2 bottle volumes of sample water and the pump flow was adjusted to prevent oxygenation due to bubbles. A subsample (500 µl) was taken without air-contact with a calibrated, gastight syringe (Hamilton, Bonaduz Switzerland) from the bottom of the bottle. After the syringe had been purged with sample several times, a sample was immediately derivatized with 50 µl monobromobimane (48 mM, Sigma) in 50 µl HEPES-EDTA buffer (pH 8, 500 mM, 50 mM) (Fahey and Newton, 1987). The samples were stored at 4 °C in the dark and were analyzed on board by reversed-phase HPLC within 8 h. A Sykam gradient controller S2000 combined with a LiChrosphere 60RP select B column (125x4 mm, 5 µm; Merck) and a Waters 470 scanning fluorescence detector were used for analysis. Eluent A was 2.5% (v/v) acetic acid pH 3.5 (adjusted with 5 N NaOH), eluent B was 100% HPLC-grade methanol, the flow

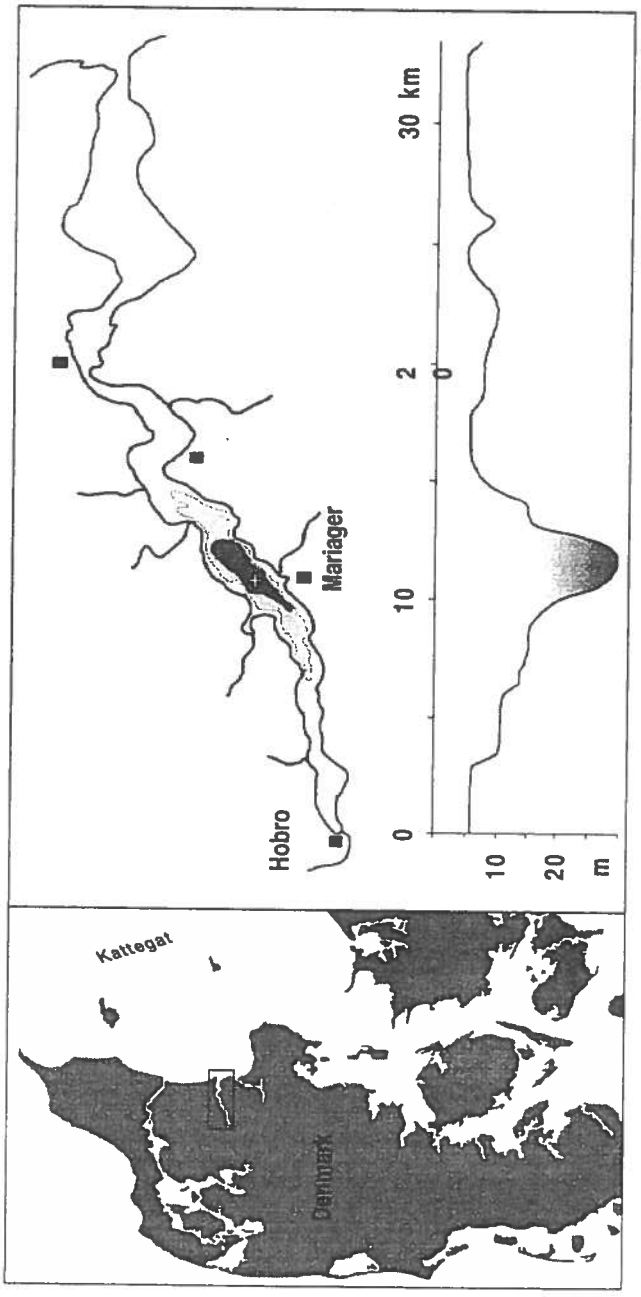

Figure 1: Mariager Fjord in northeastern Denmark is indicated by a box (left). A map of Mariager Fjord and a depth transect are shown on the right side. The main sampling location in the center of the deep basin is indicated by a white cross.

rate was 1 ml min^{-1}. A slight modification of the gradient conditions described by Rethmeier et al. (1997) was used: start 10% B, 7 min 12% B, 15 to 19 min 30% B, 23 min 50% B, 30 min 100% B, 33 min 100% B, 34 min 10% B, 39 min 10% B, injection of the next sample.

Standards for sulfite and thiosulfate were prepared in degassed Milli-Q water. No difference was observed between calibration curves with standards prepared in seawater or Milli-Q water. Detection limits for thiosulfate and sulfite were about 200 nM. The precision for repeated injection of 10 µM standards was better than ± 3% (RSD).

For zero-valent sulfur determination (S^0) an additional subsample (1ml) was taken from the same glass bottle and fixed in 100 µl 2% (wt / vol) $ZnCl_2$. With this treatment, the sulfane-S components of polysulfides are also transfomed to elemental sulfur. Elemental sulfur was then dissolved by adding 10 ml 100% HPLC-grade methanol and analyzed as S_8 by HPLC using a Sykam pump (S1100), an UV-Vis Detector (Sykam S3200), a Zorbax ODS-column (125x4 mm, 5 µm; Knauer, Germany) and 100% Methanol (LiChrosolv®, Merck) at a flow rate of 1 ml per minute as the eluant. Cyclo-octasulfur (S_8) was detected at 265 nm, the detection limit was about 1 µM and the precision was 0.5 % RSD.

Sulfate concentration in the sediment porewater was measured using non-suppressed ion-chromatography (Waters 510 pump, Waters 430 conductivity detector). Separation was made with an IC-Pak™ anion exchange column (50x4.6 mm; Waters) with a flow of 1 ml min^{-1} of 1 mM isophtalic acid in 10% methanol (pH 4.6, adjusted with saturated sodium borohydrate solution).

Metals, nutrients and DIC: For analysis of manganese, iron, inorganic nitrogen com–pounds and dissolved inorganic carbon (DIC), 50 ml water samples were pumped directly into a syringe, avoiding air contact, and filtered through N_2-flushed 25 mm Whatman GF/F glass fiber filters. Part of the filtrate was immediately fixed with 1% (vol.) of 6N HCl for later analysis of soluble Fe^{2+} and Mn (assumed to be Mn^{2+}). The filters were purged with air to remove the remaining water and stored frozen for analysis of particulate Mn and Fe. A second fraction of the filtrate was frozen for later determination of NO_3^-, NO_2^-, and NH_4^+. For determination of dissolved inorganic carbon, a third fraction was collected in 2 ml glass vials sealed with teflon coated butyl rubber septa, leaving no headspace.

Soluble Mn was determined by flame atomic absorption spectroscopy (± 2% RSD; det. limit 0.5 µM) and Fe^{2+} by complexation with Ferrozine in HEPES buffer (Sørensen, 1982; RSD ± 2%; detection limit 0.2 µM). Particulate Mn and Fe were extracted from filters by dithionite-citrate- acetic acid which preferentially dissolves free metal oxides and reduced Mn(II) and Fe(II) in carbonates and monosulfide (Thamdrup et al., 1994a). Manganese in the extracts was determined by flame AAS (RSD ± 2%; detection limit <1 µM) and iron by Ferrozine colorimetry (Thamdrup et al., 1994a; RSD ± 2%, detection limit 0.2 µM). Particulate acid volatile sulfur (AVS = FeS) and chromium reducible sulfur (CRS = FeS_2 + S^0) in the water column were determined by the two step chromium reduction technique (Fossing and

Jørgensen, 1989). Nitrite was determined as descibed by Grasshoff (1983; RSD ± 1%, det limit 0.2 µM). The concentration of NO_3^- plus NO_2^- was determined by chemoluminescence detection after reduction to NO (Braman and Hendrix, 1989; RSD ± 1%, det. limit 0.5 µM), and NO_3^- was calculated by subsequent subtraction of the NO_2^- concentration. Ammonium and DIC were analyzed by flow injection with gas exchange and conductivity detection (Hall and Aller, 1992; NH_4^+: RSD ± 2%, det limit 2 µM; DIC: RSD ± 2%). In sulfidic samples, H_2S was removed with H_2O_2 (0.5% final conc.) before DIC determination to avoid interference (Thamdrup and Canfield, 1996).

Water samples for pigment (chlorophylls, carotenoids) determination were directly pumped through a glass fiber filter (GF/F, Whatmann) and immediately frozen and stored in liquid N_2. After the cruise the samples were stored at -80 °C. 1 ml N_2-purged acetone was added to the filters and pigments were extracted for 24 h on ice in the dark. The supernatants were removed and dried in a speed-vac centrifuge (Savant SC110A). The dried pigments were taken up in 50 µl acetone and analysed. Pigments were separated on a Waters HPLC that included a 600E gradient module with system controller, a Model 991 photodiode-array detector and a Hypersil ODS-column (250x4mm, 5 µm; Knauer, Germany). Pigment identification was based on known retention times and spectra from standards. A more detailed description of the method can be found in Karsten and Garcia-Pichel (1996).

2.3 Rate determinations and light measurement

Dark $^{14}CO_2$ incorporation rate: Water samples from different depths around the chemocline were pumped directly into 50 ml glass bottles as described above. After adding 370 kBq of ^{14}C-bicarbonate (Amersham) in 5% NaCl to each sample, the bottles were incubated at the in situ temperature of 14 °C in the dark. Samples poisoned with 36% formaldehyde (1.4 % final conc.) at time 0 served as controls. After 1.5 h the incubation was stopped by adding 36 % formaldehyde (2.9% final conc.). The whole content of the bottles was filtered through a 0.45 µm cellulose filter (HAWP, Millipore). The filter was folded into a scintillation vial and fumed in a desiccator with HCl fumes for 5 minutes. The remaining radioactivity on the filters was counted in 20 ml Ultima Gold scintillation liquid on a Canberra-Packard liquid scintillation analyzer. The rates of dark CO_2-incorporation were calculated according to:

$$CO_2\text{-fixation rate} = {}^{14}\text{C-fixed} * [\Sigma CO_2] * 1.06 / \Sigma^{14}CO_2 * t \qquad (1)$$

where ^{14}C-fixed is the radioactivity counts per filter minus control, $[\Sigma CO_2]$ is the total dissolved inorganic carbon (DIC) concentration in the water at each depth, 1.06 is the correction factor for isotopic fractionation between ^{12}C and ^{14}C, $\Sigma^{14}CO_2$ is the total DIC radioactivity per bottle during incubation and t is the incubation time.

Potential sulfide oxidation rate: Water samples from 13.2 m (58.9 µM dissolved O_2), 13.5 m (<1 µM O_2) and 14.0 m (no O_2) depth were spiked with sulfide to 50 µM sulfide final concentration. The bottles were incubated at *in situ* temperature in the dark and the disappearance of sulfide under the given initial oxygen concentrations was followed.

Sulfate reduction rate: Sulfate reduction rates in the sediment were determined by whole-core $^{35}SO_4^{2-}$ incubation and subsequent single step chromium reduction (Ferdelman et al., 1997; Fossing and Jørgensen, 1989).

The sedimentation rate (ω_0) and sediment accumulation rate (r) were determined from the distribution of excess ^{210}Pb (Robbins, 1986). Gamma analysis of dried sediments for the determination of ^{210}Pb (47 keV), ^{226}Ra daughter products ^{214}Pb (295 and 352 keV) and ^{214}Bi (609 keV), and ^{137}Cs (662 keV) were performed at the University of Delaware, College of Marine Studies. Excess ^{210}Pb was calculated by subtracting the average activities obtained for ^{226}Ra daughter products ^{214}Pb and ^{214}Bi from the ^{210}Pb activity.

Light intensity was measured as PAR (photosynthetically available radiation) scalar irradiance with a Spherical Quantum Sensor which was vertically mounted on a lowering frame and connected to a LI-1000 data logger (Li-Cor). The measurements were made at 9.30 and 13.00 local time in full sunshine and with few clouds and were averaged for 20 seconds at each depth. The vertical attenuation coefficient K_d(PAR) was calculated (Kirk, 1994):

$$E_d(z) = E_d(0) \cdot e^{-K_d(PAR) \cdot z} \qquad (2)$$

where $E_d(0)$ and $E_d(z)$ are the downward irradiance just below the surface and at depth z, respectively. K_d(PAR) is the averaged vertical attenuation coefficient for the depth interval 0 to z m.

2.4 Bacterial counting

Media for MPN (most probable number) counts of sulfate- and sulfur-reducing bacteria were prepared according to Widdel and Bak (1991) and Widdel and Pfennig (1991). The sulfate reducer medium was adjusted to pH of 7.2, and supplemented with 20 mM lactate or 10 mM acetate. The elemental sulfur reducer medium was adjusted to pH 7.8, to buffer acidification during sulfur reduction, and supplemented with 5 mM acetate, the most common electron donor for sulfur-reducing bacteria of the genus *Desulfuromonas* (Widdel and Pfennig, 1991). Media were dispensed in 9 ml portions into glass culture tubes. To each tube of sulfur reducer medium, a few sulfur granules (approx. 50-100 µl volume) were added which had been autoclaved for 30 min at 112-115°C (Widdel and Pfennig, 1991). The medium for anaerobic thiosulfate oxidizers consisted of the following salts (g l^{-1}): 25 g NaCl, 1 g $(NH_4)_2SO_4$, 1.5 g $MgSO_4$ * 7 H_2O, 0.42 g KH_2PO_4, 0.3 g KCl, 0.29 g $CaCl_2$ * 2 H_2O, 0.2 g KNO_3. After autoclaving, the medium was cooled under a 90% N_2 / 10% CO_2 atmosphere and

30 ml 1 M $NaHCO_3$ solution, trace metals and vitamins were added. Sterile Na_2SO_4 solution (1.58 g in 10 ml) was added. The final pH was 7.2 ± 0.2. The medium was dispensed in 9 ml portions into glass culture tubes (Hungate tubes) and the headspace was gassed with 90% N_2 / 10% CO_2.

Triplicate MPN dilution series were inoculated with 1 ml Mariager Fjord water. The following depths were sampled: a) 13 m, just above the chemocline, oxic (220µM) with no sulfide; b) 13.65 m, exactly within the oxygen-sulfide transition zone, where the two compounds coexisted at concentrations of 10 - 40 µM; c) 14.4 m, below the chemocline, with no oxygen and substantial sulfide levels (180 µM); d) 15.9 m, deeper in the anaerobic and sulfidic water column (260 µM sulfide); e) the uppermost 5 cm of the liquid, fluffy bottom sediment at 28 m depth. Here, 1 ml sediment was homogenized with 9 ml medium and 1 ml portions were used for MPN inoculations. All samples were subsequently diluted in six 1:10 dilution steps. MPN tubes were incubated at 15 - 16 °C, the *in situ* temperature within the chemocline. The final MPN scores were reached after eighteen months of incubation. The MPNs were scored positive when microbial growth, either as turbid suspension or as bacterial clumps, coincided with sulfide production, as determined with the $CuSO_4$ test (Widdel and Bak, 1991). Standard MPN evaluation tables and 95% confidence intervals were used (APHA, 1992).

3. Results

3.1 Hydrographical parameters

Several CTD-casts were taken during a 4 day survey on Mariager Fjord. A summary of physical parameters from the water column in the central basin is shown in Fig. 2. During the first day, wind of 8 - 14 m s^{-1} led to enhanced mixing in the surface layer and caused an erosion of the pycnocline, resulting in very steep gradients of salinity, temperature, and chemical parameters (Fig. 2a). From day 2 to 4 the wind was weak (0 - 3.3 m s^{-1}) and a relaxation of the pycnocline was observed (Fig. 2b,c) The upper boundary of the pycnocline moved upwards from 13.8 m (day 1) to about 11.7 m (day 4). Whereas the profiles of temperature and salinity from day 1 and 4 were rather smooth, profiles from day 2 and 3 (data not shown) were irregular and indicated enhanced disturbance at the pycnocline. On the same days, step-like structures in the temperature profile together with the temperature-salinity (T/S) plots indicate the intrusion of more saline and cooler water from the outer parts of the fjord or the sea (Fig. 2e). Water at a depth of 14.4 m was only slightly affected and the bottom water was not affected by the intrusion. In Table 1 data on wind speed, the depth of the pycnocline, the chemocline, and the extension of the oxygen-sulfide interface are summarized.

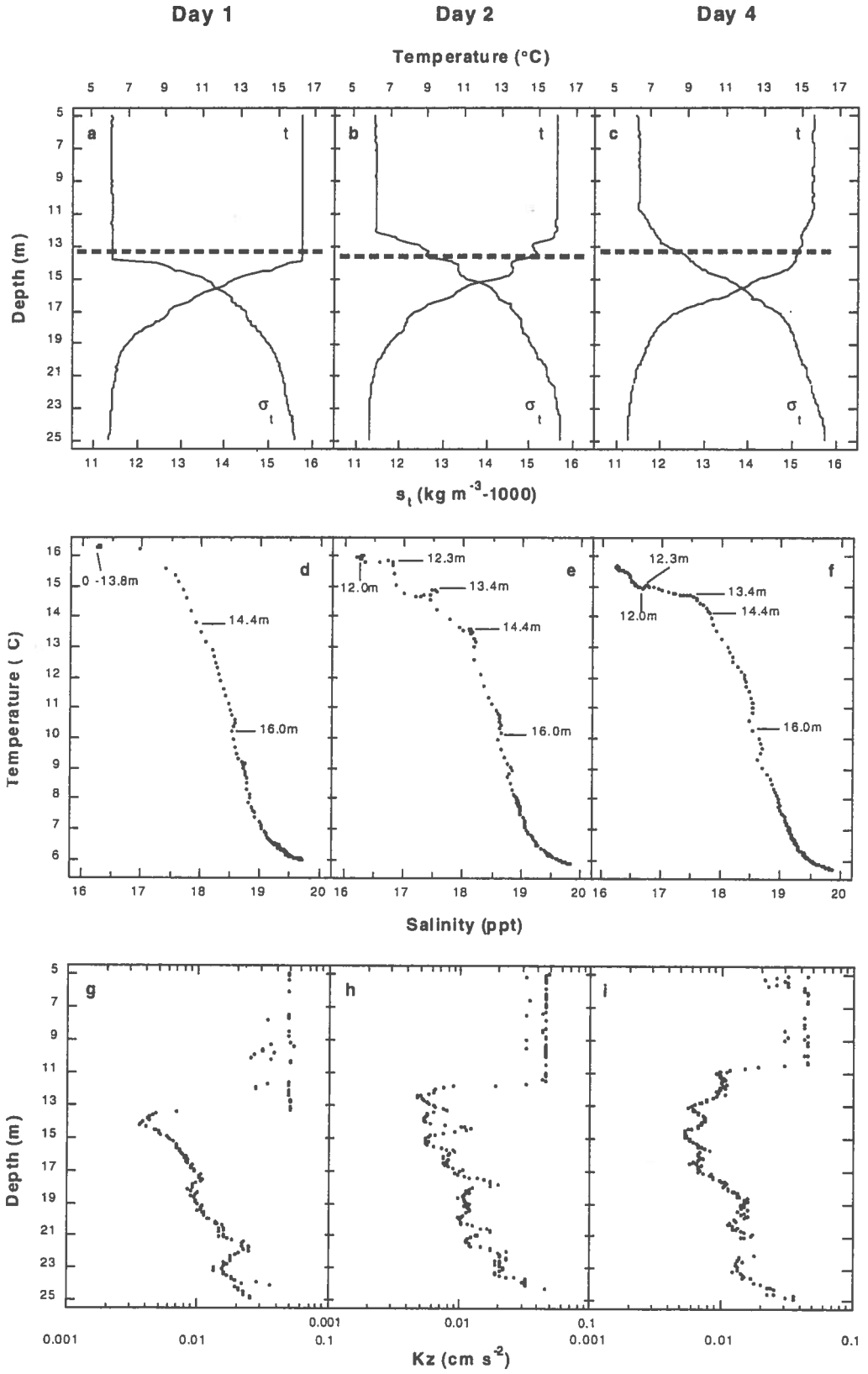

Figure 2: Compilation of physical parameters of Mariager Fjord during the survey on day 1, day 2, and day 4 (13-16 September 1996). Depth profiles of density and temperature are depicted in row 1. The hatched line indicate the location of the oxygen-sulfide interface. Temperature vs. salinity (T/S) plots and calculated vertical eddy diffusivities are shown in row 2 and 3 respectively.

Table 1: Physical and chemical characteristics of the different profiles and results of potential sulfide oxidation rate calculations under the actual *in situ* conditions.

Profile	Day	Wind	Pycnocline	Chemocline				Sulfide oxidation rate in chemocline			
			depth[a)]	depth [b)]	extension [c)]	O_2 [d)]	H_2S [d)]	total [e)]	chemical [f)]	biological [g)]	biological
#	#	($m\ s^{-1}$)	(m)	(m)	(m)	(µM)	(µM)		($\mu mol\ l^{-1}\ d^{-1}$)		(% of total)
1	1	8-14	13.8	13.4	1.0	62.8	56.3	6.2	142.2	-	-
5	2	0-3.3	11.8	13.8	1.0	50.5	7.8	6.2	15.8	-	-
6	3	0-3.3	11.8	13.6	0.3	5.1	13.3	20.5	8.5	12.0	58.5
7	3	0-3.3	11.7	13.4	0.15	16.5	7.4	41.1	4.9	36.2	88.0

a) Upper boundary of pycnocline. b) Depth of oxygen and sulfide gradient intersection. c) Depth interval of oxygen and sulfide coexistence. d) Average oxygen and sulfide concentrations in the chemocline. e) Calculated by dividing the sulfide flux to the chemocline by the volume of the chemocline (on a m^2 base). f) Calculated for average oxygen and sulfide concentrations and *in situ* conditions (t = 15°C, S = 17 ‰, pH 7.8, 1µM Fe^{2+}). These calculations represent *theoretical* chemical sulfide oxidation rates. g) Biological sulfide oxidation rate calculated by the difference between the actual rate and the theoretical chemical rate. Since the chemical rate is a potential rate, the obtained values for biological sulfide oxidation represent minimal estimates.

3.2 Chemical parameters

In profile 1 from day 1 (Fig. 3), oxygen was evenly distributed in the mixed water body with concentrations of ca 250 µM. Below a depth of 13 m the oxygen concentration dropped steeply to the chemocline at 13.4 m. Below the chemocline the sulfide concentrations steadily increased and reached 662 µM at 27 m depth. In contrast to an earlier study (Ramsing et al., 1996), there was no indication of sulfide oxidation below the chemocline.

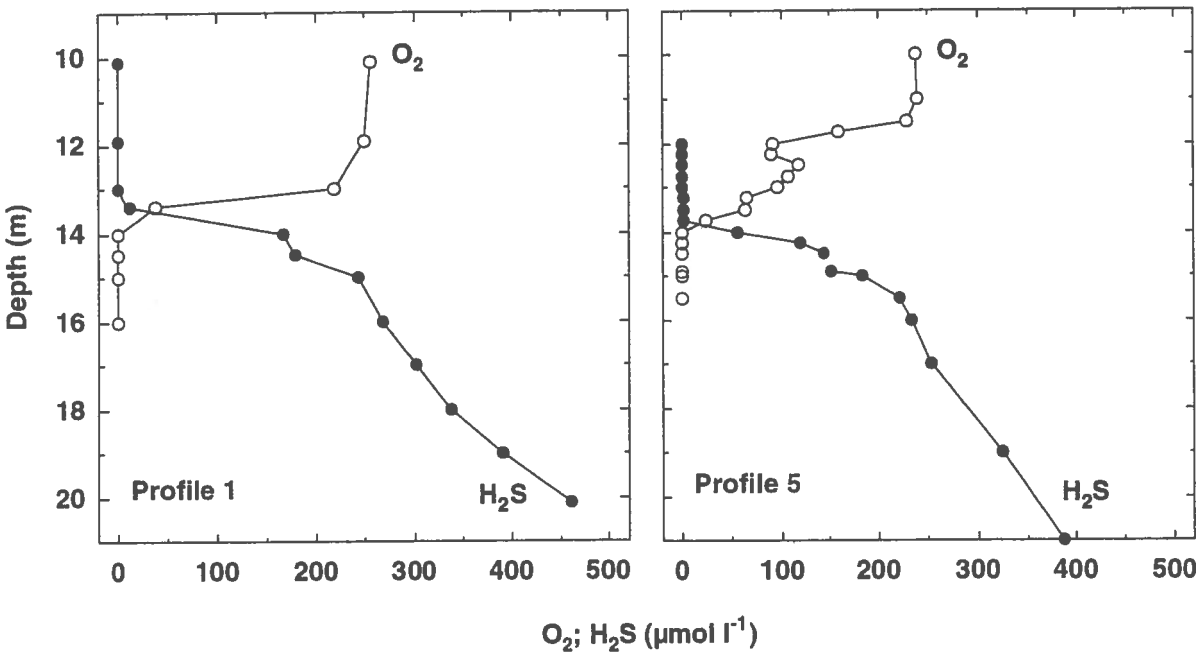

Figure 3: Depth profiles of oxygen and sulfide profiles during high wind energy input (profile 1) and the relaxation phase (profile 5).

During this first day, the chemocline and the pycnocline coincided (Fig. 2a), and an overlap of about 1 m of oxygen and sulfide was observed (Tab. 1). Although the pycnocline moved upwards by 2.1 m during the survey, this was not the case for the chemocline which remained at 13.4 - 13.8 m depth. However, the extension of the oxygen-sulfide zone and the concentrations of coexisting sulfide and oxygen varied significantly (Table 1).

The oxygen profiles from day 2 (profile 5) were less smooth than from day 1 and showed secondary maxima at 12.5 m and 13.5 m (Fig. 3). Also profiles of other chemical species showed secondary peaks above the chemocline (Fig. 4).

Nitrogen compounds: Ammonium, nitrite, and nitrate were determined for profile 5 (Fig. 4a) and 7 (not shown). In the hydrodynamically influenced profile 5, two zones of enhanced nitrification were observed indicated by nitrite and nitrate peaks.

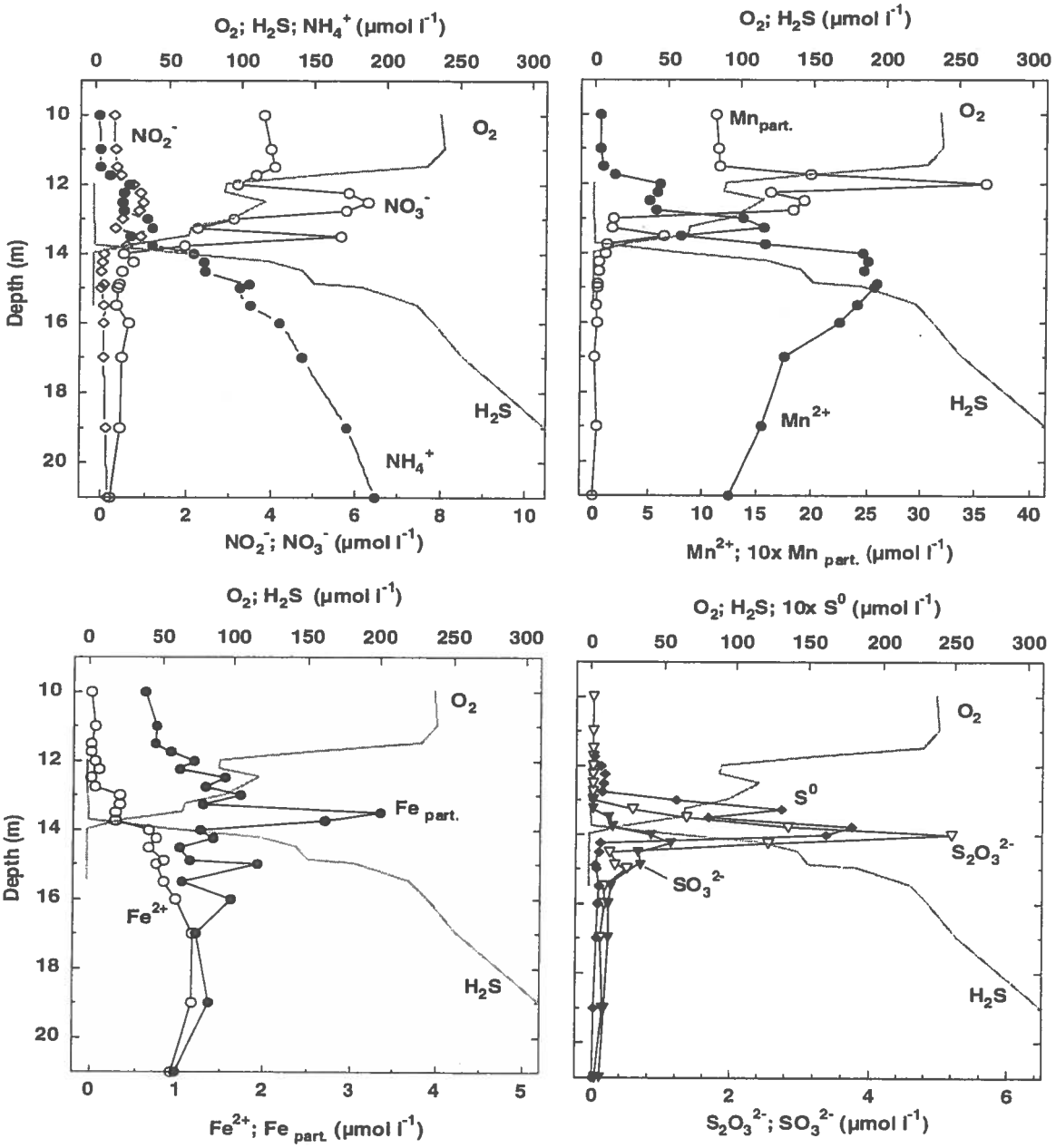

Figure 4: Depth profiles of nitrogen compounds (A), manganese (B), iron (C) and sulfur intermediates (D). Data are from profile 5.

In the lowest zone, at about 13.5 m with <60 µM dissolved oxygen, nitrification and denitrification were seemingly tightly coupled. Thus, the concentration of ammonium dropped steeply but the observed formation of nitrite and nitrate could only partially account for this loss, indicating that the produced nitrite and nitrate were further reduced to dinitrogen. In the upper zone at 12.75 m and above, only nitrification was taking place and nitrite and nitrate balanced the minimum in ammonium. Above 11.75 m ammonium had been oxidized to a background level of 2.9 µM, nitrite reached 0.3 µM and nitrate 3.8 µM. In profile 7 all the

gradients were smoother and only one nitrification zone starting at about 13.4 m was observed (not shown).

Manganese and iron: A production horizon for particulate manganese, presumably MnO_2, was located well above the chemocline at 12.0 m (Fig. 4b). The maximal concentration of 3.6 µM Mn_{part} rapidly decreased towards the chemocline, and below 15 m particulate manganese was not detected. The main reduction zone for MnO_2 was located just below 14 m as indicated by the broad and high (25.6 µM) peak of dissolved reduced manganese. Below 15 m the concentrations of Mn^{2+} decreased with depth, suggesting that the sediment of Mariager Fjord is a sink for reduced manganese which might be buried in the form of MnS (albandite) or $MnCO_3$ (rhodochrosite) (Lewis and Landing, 1991).

The maximal particulate iron concentration of 3.3 µM was found just above the chemocline at 13.5 m (Fig. 4c). Particulate iron at 14 m and above presumably consisted of iron(III)-oxyhydroxides (FeOOH) as no iron-sulfide (FeS) was detected by AVS distillation (data not shown). Below the chemocline, Fe_{part} mainly consisted of FeS and Pyrite (FeS_2) as indicated by AVS and CRS distillation. In contrast to manganese, the concentration of dissolved iron below the chemocline was low and never exceeded 1.2 µM as iron was continuously removed from the water column by precipitation and sedimentation of sulfides. Whereas Fe^{2+} was not detectable at 13.2 m and above Mn^{2+} was still found in concentrations of about 7 µM, which demonstrates the much faster oxidation of reduced iron compared to manganese. In contrast to profile 5, no peak of FeOOH was present in the hydrodynamically less influenced profile 7 taken one day later (not shown).

Sulfur compounds: The profiles of sulfide oxidation products from profile 5 are shown in Fig. 4d. Sulfite, thiosulfate, and relatively high concentrations of zerovalent sulfur (S^0) were detected in the chemocline. S^0 reached a concentration of 17.8 µmol l^{-1} at 13.75 m and thiosulfate had a sharp peak of 5.2 µM at 14 m depth. Below 15 m, thiosulfate and sulfite concentrations were very low and showed no trend with depth. The average concentrations for these values were 0.16 ± 0.13 µM (n=9) for thiosulfate and 0.18 ± 0.06 µM (n=8) for sulfite.

The results of a more detailed profile from the chemocline (profile 6) are shown in Fig. 5. In contrast to profile 5 a day earlier, lower concentrations of thiosulfate (2 µM) and elemental sulfur (11 µmol l^{-1}) were measured. In the upper part of the chemocline (< 13.65 m) thiosulfate was particularly low (< 0.1 µM) and sulfite was not detected at all.

3.3 Direct rate measurements

Maximal rates of dark $H^{14}CO_3^-$ fixation were observed at the lower border of the chemocline (Fig. 6). The integrated areal uptake rate was 10.4 mmol m^{-2} d^{-1} (13.3 - 13.6 m). The cause of the elevated values below the chemocline is not clear. The higher values could indicate a zone of bacterial disproportionation of sulfur intermediates (Finster et al., 1998) or autotrophic sulfate reduction with H_2 (Widdel and Bak, 1991).

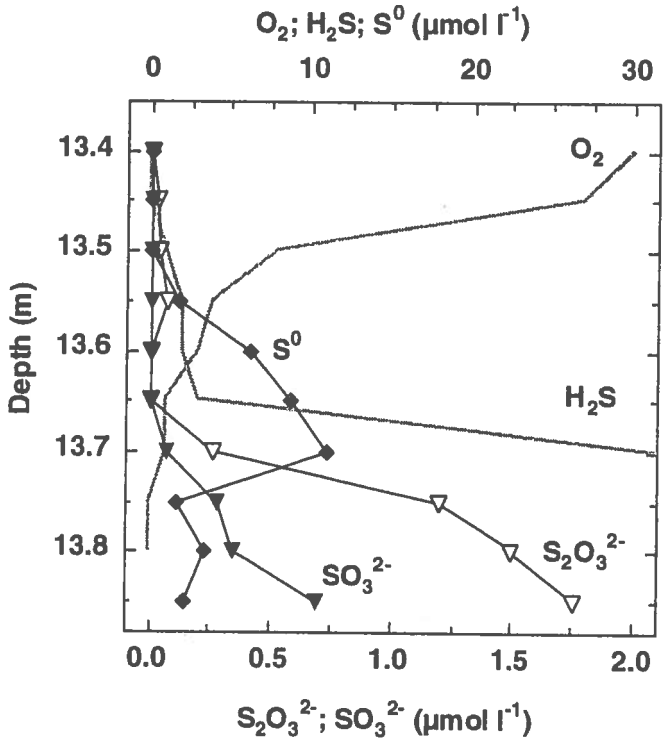

Figure 5: Detailed profiles of sulfur intermediates in the chemocline (Profile 6).

The bacterial sulfate reduction rate (SRR) in the sediment integrated over 17 cm varied strongly (4.7, 7.4, and 17.3 mmol m^{-2} d^{-1}), but the sedimentary sulfate profiles were very similar in the different cores. In order to obtain a further estimate of the SRR, we also calculated it from the sulfate gradient in the sediment. Assuming that in sediments from euxinic environments, sulfide oxidation is unimportant, the sulfate flux into the sediment should reflect the SRR. The areal SRR calculated from the sulfate profile at the sediment-water interface was 6.65 mmol m^{-2} d^{-1}. This agrees well with the two lower values from the tracer incubations. Assuming that there is no sulfide oxidation in the sediment, the SRR minus the amount of buried reduced sulfur equals the sulfide flux into the water column. Sulfur burial was 8.1% of the sulfate influx (see below) and the flux of sulfide into the water column was therefore 91.9% or 6.1 mmol m^{-2} d^{-1}.

The potential sulfide oxidation rates in the water column, which were determined by adding sulfide to water samples, showed that in samples without dissolved oxygen (13.5 m, 14 m) no significant sulfide oxidation took place within 27 h of dark incubation (data not shown). In the sample from 13.2 m depth, which had an initial O_2 concentration of 58.9 µM, the added sulfide disappeared linearly at a rate of 0.216 µM min^{-1} which is about 15 times faster than the expected rate for a purely chemical oxidation (0.015 µM min^{-1}) under the same starting conditions (Fig. 7).

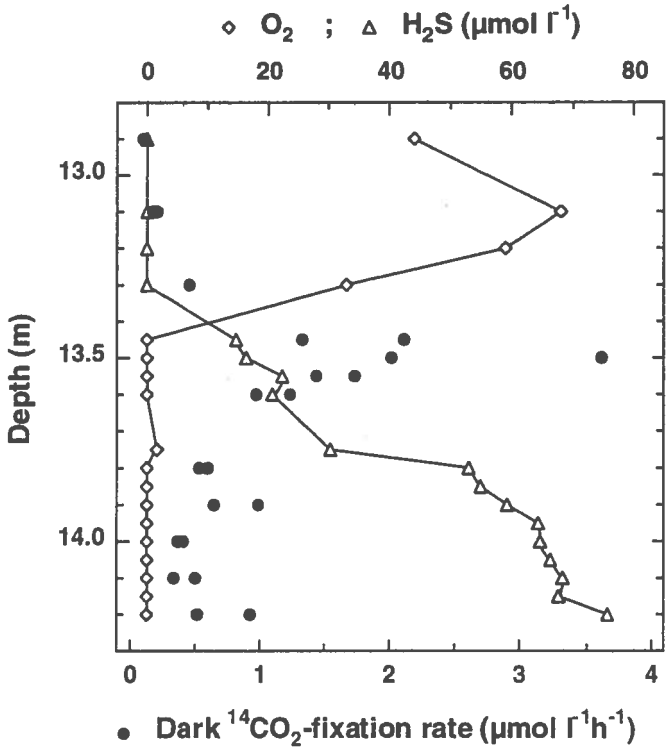

Figure 6: Dark $^{14}CO_2$ incorporation rate in the chemocline (Profile 7).

The chemical rate was calculated according to the rate law (Zhang and Millero, 1994):

$$\frac{d[H_2S]_T}{dt} = k[H_2S]_T[O_2] \qquad (3)$$

where k (M^{-1} h^{-1}) is the rate constant, $[H_2S]_T$ is the concentration of total sulfide species and $[O_2]$ is the oxygen concentration. The rate constant k (= 299 M^{-1} h^{-1}) was recalculated for the *in situ* conditions at the chemocline (temperature: 15°C, salinity: 17 ppt, pH 7.8) and the presence of 1.1 µM Fe(III) as catalyst and oxidant was also included (Millero et al., 1987a; Zhang and Millero, 1994). However, due to the limited reservoir of O_2 in the closed bottles the oxidation of H_2S was not complete and about 2 µM sulfide remained until the end of the experiment. Mass and electron balances suggest that zerovalent sulfur was the main oxidation product in the incubation experiments which is, in agreement with the fast disappearance rate, consistent with bacterially mediated sulfide oxidation.

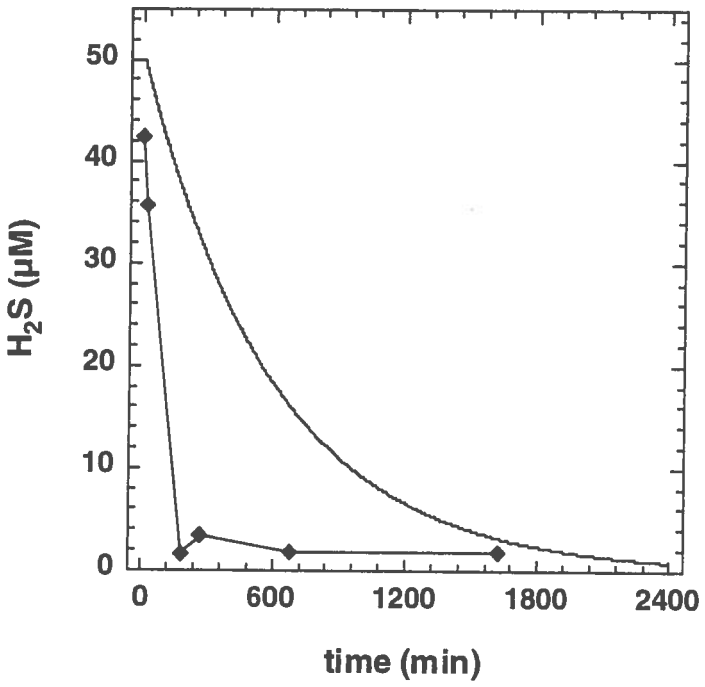

Figure 7: Comparison of the potential sulfide oxidation rate determined in closed bottle experiments with water samples from 13.2 m depth (♦) and the modeling results for the chemical oxidation under the same conditions.

3.4 Flux balances

To quantify the main redox reactions in the chemocline and to evaluate their relative significance for the dark CO_2-uptake rate or the mineralization of organic matter, the concentration gradients of the different chemical species were used to calculate the vertical fluxes in the water column:

$$F_z = -K_z \frac{dC}{dz} \qquad (4)$$

where F_z is the vertical flux, K_z is the vertical eddy diffusivity and dC/dz is the concentration gradient. K_z values for the lower chemocline and the deep water were estimated by dividing the sulfide flux (from the SRR) by the sulfide gradient. This resulted in calculated vertical eddy diffusivities of 0.0078 $cm^2 s^{-1}$ and 0.023 $cm^2 s^{-1}$ for the lower chemocline and the bottom layer, respectively. The eddy diffusivities are at least 2 orders of magnitude higher than molecular diffusion coefficients, and therefore the same K_z values can be used for all the different chemical species.

The vertical eddy diffusivity in a water column is an inverse function of the buoyancy frequency (Brunt-Väisälä frequency), a measure of vertical stability, i.e. the more stable the water body, the lower the vertical eddy diffusivity and hence the fluxes. To check if we could use the same diffusivities for compounds diffusing from above to the chemocline we estimated K_z values for other depths using the empirical relation (Gargett, 1984; Lewis and Landing, 1991):

$$K_z = a_0 \left(-\frac{g}{\rho_z} \frac{d\rho}{dz} \right)^{-0.5} \qquad (5)$$

where g is the gravitational constant, ρ_z is the average density at a given depth, and $d\rho/dz$ is the density gradient at that depth. The factor a_0 is a system-specific constant and a measure of the average input of energy to the internal wave system. The a_0 value lies between 0.001 $cm^2\ s^{-2}$ and 0.0001 $cm^2 s^{-2}$ for the open ocean and completely restricted basins, respectively, and has to be estimated in most cases (Lewis and Landing, 1991; Gargett, 1984). In our case, we solved for the a_0 using K_z from the SRR and obtained an a_0 of 0.0005. In comparison to earlier studies in the Black Sea (a_0 = 0.0004 $cm^2\ s^{-2}$; Landing et al., 1991; Lewis and Landing, 1991) and Mariager Fjord (a_0 = 0.0002 $cm^2\ s^{-2}$; Ramsing et al., 1996) this is a relatively high value for a_0 and suggests that the basin is moderately open.

The results from the flux calculations for redox sensitive elements are compiled in Table 2. Profile 7 was less disturbed by mixing processes than profile 5 and was therefore used for the flux calculations. The main electron donors diffusing from the sediment to the chemocline are sulfide and ammonium with 6.11 (67.8%) and 1.95 mmol $m^{-2}\ d^{-1}$ (21.6%), respectively. The molar ratio of sulfide to ammonium (= 3.16) was, in contrast to the findings of Ramsing et al. (1996), in good agreement with the Redfield-ratio (Froehlich et al., 1979) for the degradation of organic matter under sulfate reducing conditions. For profile 5 the sulfide-ammonium ratio was similar (3.12) for the higher concentrations (> 150 µM) but was only 2.45 at lower concentrations. This deviation from the Redfield stoichiometry could either be due to additional ammonium production in the lower chemocline (coupled to the oxidation of sulfide or organic matter) or due to enhanced sulfide oxidation during transient mixing. In profile 7 the fluxes of electron donors from the sediment and the fluxes of oxidants, mainly oxygen (15.79 mmol $m^2\ d^{-1}$ = 87.2%), are well balanced. The small deficiency of electron donor equivalents of 5.68 mmol $m^2\ d^{-1}$ (7.8%) is probably within the error of the flux calculations. Alternatively, sulfate reduction (i.e. sulfide production) in the chemocline could account for the missing electron equivalents.

Table.2: Mass and electron fluxes of reductants and oxidants in the water column of Mariager Fjord (Profile 7). Fluxes (positive = upwards, negative = downwards) were calculated for a vertical turbulent diffusion coefficient of K_z =0.0078 $cm^2 s^{-1}$.

		Electron acceptor					Electron donor				Balance
		O_2	NO_3^-	NO_2^-	Mn_{part}	Fe_{part}	H_2S	NH_4^+	Mn^{2+}	Fe^{2+}	
Mass Flux	mmol $m^{-2}d^{-1}$	-15.79	-1.25	-0.40	-0.93	n.d.	6.11 [a]	1.95	1.10	0.07	
Transferable e^-	n [b]	4	5 [c]	3 [c]	2	1	8	8	2	1	
Electron Flux	mmol $e^- m^{-2}d^{-1}$	-63.16	-6.25	-1.2	-1.86	n.d.	48.88	15.60	2.20	0.07	-5.68

[a] Based on sulfate reduction rate measurements in the sediment. For further details see text; [b] Number of electrons that can be donated or accepted by a given compound under the assumption of complete oxidation or reduction respectively; [c] Denitrification to N_2 is assumed.

Due to rapid reoxidation, sulfide produced here might not be apparent from the sulfide gradient. Under the assumption that both nitrite and nitrate served only as electron acceptors and were reduced to dinitrogen, their contribution to the total electron balance was about 10.2 %. Manganese and especially iron are only minor contributors to the total electron budget. For particulate iron it was not possible to calculate a downward flux, because there was no clear gradient. However, the downward flux of particulate (oxidized) iron had to be at least equal to the upward flux of dissolved Fe^{2+} (0.07 mmol m^{-2} d^{-1}). The downward flux of reactive iron is even better constrained by the Fe(II) burial in the sediment. Using the burial rate of sulfur (0.20 mol S m^{-2} yr^{-1}, see next section) and assuming that iron is deposited as pyrite (FeS_2), one can calculate a minimal input of reactive iron of 0.27 mmol m^{-2} d^{-1}.

The contribution of manganese to the total electron balance was about 3%. The up- and downward fluxes are almost balanced although the downward flux of manganese was lower. As for iron, the downward flux of manganese should be equal to or higher than the upward flux of the dissolved, reduced species. Therefore, particle transport must exceed the diffusive transport of dissolved compounds and the calculated downward fluxes of oxidized iron and manganese are underestimated.

3.5 Sulfur and carbon budget

The sedimentation rates were determined from ^{210}Pb and ^{137}Cs profiles. The distribution of excess ^{210}Pb in the non-bioturbated sediments from the central basin of Mariager Fjord showed an exponential decrease with depth (data not shown). A high sedimentation rate (ω_0) of 1.4 cm yr^{-1} and a sediment accumulation rate (r) of 380 g (d. w.) m^{-2} yr^{-1} were calculated (Robbins, 1986). A broad peak of ^{137}Cs resulting from Chernobyl fallout (1987) was observed at a depth of 5 to 7 cm (data not shown) which confirms the above-calculated data. The organic carbon content decreased from 18.8% at the sediment surface to a rather constant average value of 12.1 ± 0.8 % (n = 12) between 6 and 30 cm depth. The average sulfur content was 1.72 ± 0.27 % (n = 15). Burial rates for organic carbon and total sulfur were 3.83 mol C m^{-2} yr^{-1} (380*0.121/12.01) and 0.20 mol S m^{-2} yr^{-1} (380*0.0172/32.06). Simplified budgets for organic carbon and sulfur are shown in Fig. 8.

The mean primary productivity in the mixed layer during the period 1988 - 1997 was 70.3 mol C m^{-2} yr^{-1} (Andersen et al., 1998). The relative contribution of nitrate and manganese reduction to the mineralization of organic matter was estimated from the flux calculations (Table 2) under the assumption that these electron acceptors were exclusively reduced by organic matter and not by sulfide. The values for organic matter mineralization via nitrate and manganese reduction (Fig. 8) represent therefore upper estimates. Sedimentary methane production rates of 0.3 and 1.5 mmol m^{-2} d^{-1} were reported by Fenchel et al. (1996). Since we do not have data for methane production, an intermediate value of 0.9 mmol m^{-2} d^{-1} was included in the budget.

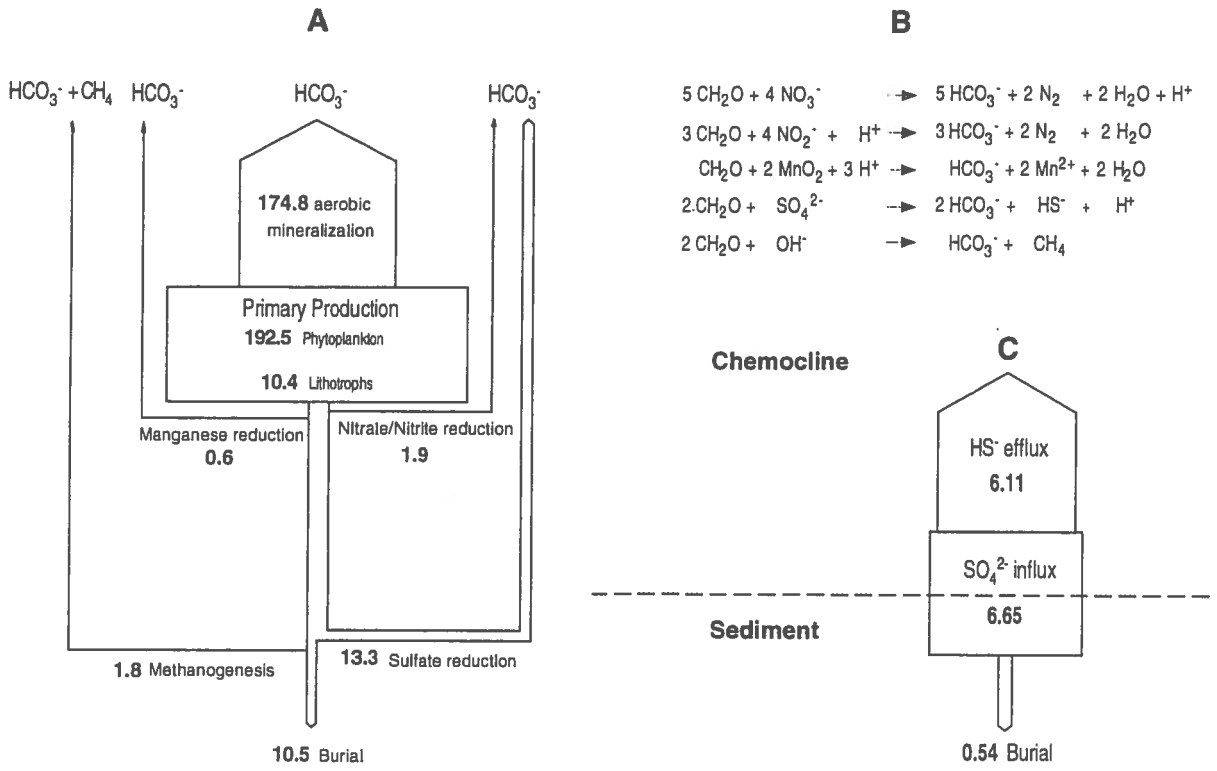

Figure 8: Carbon budget for the central deep basin of Mariager Fjord, in mmol C m^{-2} d^{-1} (A). The amount of organic matter degraded by the different mineralization processes is calculated according to the stoichiometry (B) and the corresponding flux of oxidant (Table 2). Values for primary production by phytoplankton and methanogenesis are taken from Andersen et al. (1998) and Fenchel et al. (1996), respectively. C) Simplified sulfur budget for for the same station, in mmol S m^{-2} d^{-1}. For further details see text.

3.6 Light measurement and biological parameters

The light intensity in the water column decreased exponentially with depth and was <0.003% of surface intensity in the chemocline at 13.5 m (Fig. 9). This corresponds to a quantum flux of 0.12 µE m^{-2} s^{-1}. The vertical attenuation coefficient (K_d (PAR)) for downward quantum irradiance of PAR calculated for each depth was highest close to the surface (0.98 m^{-1} at 2 m) and decreased with depth. The average K_d(PAR) for the whole water column was 0.72 m^{-1}.

Pigment determination: Neither bacteriochlorophylls nor carotenoids (e.g. okenone, chlorobactene, spirilloxanthine, isorenieratene) could be detected in water samples from the chemocline and below. Small peaks of degradation products of (bacterio-)chlorophylls and carotenoids were observed, but affiliation to precursor pigments was not possible.

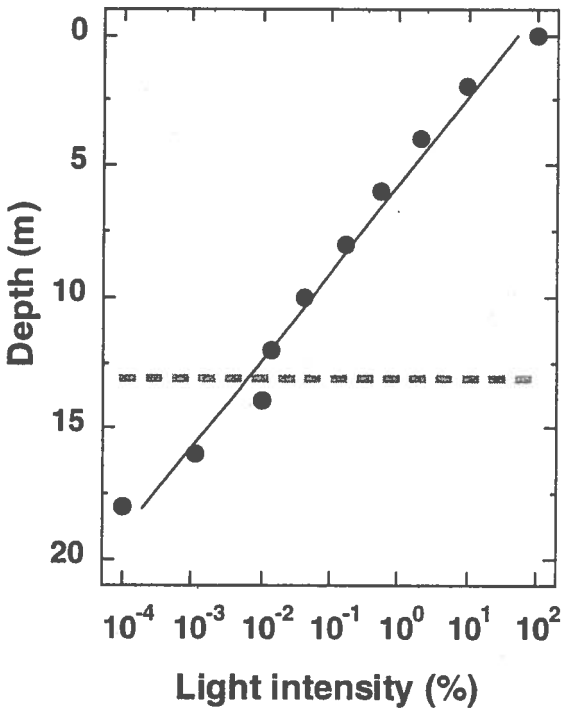

Figure 9: Light intensity measured as PAR and expressed as percent of the intensity at 0.2 m above surface. The hatched line indicates the location of the chemocline.

MPN counts (Table 3): A gradual, but statistically insignificant, increase of the lactate-utilizing sulfate reducing bacteria (SRB) at the chemocline contrasts with a sharper and significant increase by at least one order of magnitude for acetate oxidizing sulfate- and sulfur reducing bacteria. Similar results were obtained during a previous cruise to Mariager Fjord in August 1993 (Teske et al. 1996). The MPN counts of sulfur reducers were intermediate between lactate- and acetate-utilizing SRB. Whereas incomplete oxidizing SRB appear to be the dominant group of sulfate reducers in the water column and are also found above the chemocline, acetate oxidizing sulfur- and sulfate-reducers are more important in the sediment. Greenish-brown cell clusters preliminary identified as *Chlorobium* spp. grew in sulfur reducing MPN's from all depths, which were incubated in the light, indicating the presence of an anoxygenic phototroph population of at least the size of the sulfur reducing population.

Tab. 3: Most probable number counts of sulfate and sulfur reducing bacteria per ml water. The 95 % confidence intervals are given in parentheses. Lactate and acetate were the added substrates.

Depth	Sulfate reducers		Sulfur reducers
m	Lactate	Acetate	Acetate
13.00	93 (15 - 380)	1 (0.3 - 3.6)	9.3 (1.5 - 38)
13.65	93 (15 - 380)	1 (0.3 - 3.6)	4.3 (0.7 - 21)
14.40	240 (26 - 1300)	43 (7 - 210)	75 (14 - 230)
15.90	159 (30 - 440)	24 (3.6 - 130)	93 (15 - 380)
Sediment surface (ca. 26)	43'000 (7'000 - 210'000)	93'000 (15'000 - 380'000)	93'000 (15'000 - 380'000)

4 Discussion

4.1 Water column dynamics

Three different stages in the dynamics of the water column of Mariager Fjord were identified. Stage A (Fig. 2a,d,g) was characterized by strong energy input by wind which led to increased turbulent mixing and deepening of the mixed layer. However, the density and T/S-plots indicate quasi-steady-state conditions in the water column with a strong stratification under continuous energy input. Wind forces could also increase the export of light, brackish surface water to the open sea (Fenchel et al., 1995). Stage B (Fig. 2b, e, h) was clearly characterized by transient non-steady-state conditions. Diminished kinetic energy in the mixed layer allowed the pycnocline to relax, and an influx of relatively dense, oxygenated water from the sea was observed. We do not know, whether the intruding water compensated for a possible increased efflux of surface water during stage A or whether the intrusion was caused by events in the Kattegat. However, the intrusion layer was located between 12 and 15 meter and strongly influenced the biological and chemical processes at the chemocline (13.4 m), since internal waves and shear caused by the intrusion lead to mixing and enhanced vertical transport in the water column (Mann and Lazier, 1996). During stage C (Fig. 2c,f,i) the water column approached a new steady state under low energy input conditions. However, the different CTD casts from that day showed small variations in the density and temperature profiles which

indicates the presence of internal waves (data not shown). The stratification was weaker than during stage A as shown by the higher K_z values.

Our data show that, although a shallow and long channel connects the central basin of Mariager Fjord with the Kattegat, strong water exchange can be induced by wind speeds of only 8 - 14 m s^{-1}. The upper water column and chemocline of Mariager Fjord is more dynamic than assumed in an earlier study (Ramsing et al., 1996). Within a few days we observed a decrease in the surface water temperature and a slight increase in the surface water salinity, caused by mixing with colder, more saline water. Intrusions of relatively dense, oxygenated water, as observed on days 2 and 3, are frequently observed in Mariager Fjord (F. Andersen pers. comm.). However, as we also observed, such events are only apparent for a short time and can therefore easily be overseen.

4.2 Effect on chemical speciation and distribution

A clear effect of the in intrusion could be seen in the oxygen profiles (Fig. 3 and 4). Instead of oxygen concentrations steeply decreasing towards the chemocline, an intermediate maximum was observed at 12.5 m. The oxygen concentration within the intrusion was not as high as in the upper mixed layer which indicates that mixing with oxygen-deficient water could have occurred already during inflow. This was probably caused by shear due to the strong horizontal component in the movement of the intrusion layer. By this process anoxic, reduced species-containing water from below the chemocline was mixed with the intruding water leading to a quasi continuation of the concentration profiles of reduced species in the intrusion layer.

According to a simple diffusion calculation based on the measured density stratification, it would take two weeks for a given chemical compound to cross an intrusion layer of 1 m extension by eddy diffusion alone ($t = (\text{distance})^2 / K_z = 100^2\ cm^2 / 0.0078\ cm^2\ s^{-1} = 14.8$ d; Mann and Lazier, 1996). This is much too slow to explain the observed distribution pattern of reduced species within the intrusion, and other transport mechanisms like shear must be responsible. Within the intruding water mass, entrained reduced compounds were oxidized rapidly, which led to peak concentrations of the oxidized species, NO_3^-, NO_2^-, Mn_{part}, and Fe_{part}. Mixing and oxidation were very pronounced in the concentration profile of Fe_{part} (Fig. 4c) which reached a maximum of 3.3 µM at 13.5 m. Fe^{2+} reacts rapidly with oxygen with half-lives of only 1.8 min under air saturation and 25 min at 50 µM O_2 (Millero et al., 1987b). No peak of oxidized particulate iron was observed in later profiles, probably due to reduced mixing. The reduced species, NH_4^+, Mn^{2+} and S^0, which are less reactive with oxygen, were observed as shallow as 12 m or even 11.75 m water depth. This points to a fast vertical transport of formerly anoxic, nutrient rich water into the mixed surface layer which could greatly stimulate primary productivity in the euphotic zone.

4.3 Sulfide oxidation

The sulfur intermediates (S^0, $S_2O_3^{2-}$, SO_3^{2-}) exhibited maximum concentrations in or very close to the chemocline. The main production horizon for these compounds was apparently the oxygen-sulfide interface. They may then be further oxidized to sulfate above the chemocline or may be reduced or disproportionated under anoxic conditions below the chemocline. So far, only very limited measurements of sulfur intermediates in anoxic basins are available and most of the data do not include sulfite (Table 4). In this study we observed high concentrations of sulfur intermediates in the chemocline, which contrasts the results of an earlier study in Mariager Fjord (Ramsing et al., 1996) where a maximum of 0.6 µmol l^{-1} S^0 was measured. In that study, thiosulfate was not detected at all which might have been due to the low sensitivity of the method used. We propose that the high concentrations of S^0, $S_2O_3^{2-}$ and SO_3^{2-} of 17.8 µmol l^{-1}, 5.2 µM and 1.2 µM respectively in our study were mainly the result of disturbance by mixing and oxygenation. The elevated concentrations were a transient phenomenon as suggested by steadily decreasing peak concentrations during our survey. One day later, maxima of 11 µmol l^{-1} S^0, 2 µM $S_2O_3^{2-}$ and 0.7 µM SO_3^{2-} were determined. Two days later, thiosulfate and sulfite maxima were both 1.3 µM (no S^0 data).

Provided that all the observed sulfur intermediates were formed in the chemocline, the distribution of these compounds above the chemocline imply that elemental sulfur, which is present at shallower depths, is oxidized more slowly than thiosulfate and especially sulfite. Sulfite is rapidly oxidized to sulfate in presence of oxygen and trace metals, which act as catalysts (Fe^{2+}) or additional oxidant (Fe(III)) (Millero, 1991a; Zhang and Millero, 1991). Although sulfite may serve as a substrate for growth in some *Thiobacillus* species (Beffa et al., 1993) and heterotrophic sulfur oxidizing bacteria (Sorokin and Lysenko, 1993), the ecological significance of this process is unclear in view of the very low concentrations of SO_3^{2-} found in nature and the fast kinetics of chemical sulfite oxidation. On the other hand, thiosulfate (Millero, 1991a) and elemental sulfur are relatively stable under oxic conditions in the absence of bacteria. By means of molecular techniques it can be shown that heterotrophic bacteria capable of oxidizing reduced sulfur compounds (Sorokin and Lysenko, 1993) form a numerically dominant community in coastal waters (González et al., 1999).

Experimental studies have shown that sulfite, thiosulfate and sulfate are the main products of chemical sulfide oxidation (Zhang and Millero, 1993b). We observed that elemental sulfur was by far the most abundant sulfur intermediate. Under laboratory conditions, S^0 mainly forms when the ratio of sulfide to oxygen is high (Chen and Morris, 1972; Sorokin, 1970). Trace metals are known to influence the sulfide oxidation rate as well as the products formed. For example, nickel (Kotronarou and Hoffmann, 1991) and lead (Zhang and Millero, 1993b) enhance the formation of S^0. In Mariager Fjord, however, manganese and iron are quantitatively the most important metals. Neither the presence of their reduced nor oxidized forms stimulate S^0 formation under oxic

Table 4: Concentration maxima and methods of sulfur intermediate measurements in euxinic water bodies.

Site	S^0 (μM)	$S_2O_3^{2-}$ (μM)	SO_3^{2-} (μM)	Maxima[a)]	Method[b)]	Reference
Black Sea	0.06	(<0.05)[c)]	(<0.05)	C	1	Luther et al., 1991
	n.d.[d)]	2.9	2.1	D	2	Vairavamurthy & Mopper, 1990
	0.2	n.d.	n.d.	C	3	Jørgensen et al., 1991
	3.4	2.8	n.d.	D	4	Volkov, 1991
	n.d.	0.09	(<0.05)	C	6	Zopfi, 1997, unpubl.
Cariaco Trench	n.d.	14	2	C	5	Tuttle & Jannasch, 1979
	n.d.	1.2	1.8	D	2	Zhang & Millero, 1993a
	0.4	n.d.	n.d.	C	4	Hastings & Emerson, 1988
Framvaren Fjord	(<1)	n.d.	n.d.	-	4	Jacobs et al., 1985
	n.d.	7/35	3	C/D	2	Millero, 1991b
Mahoney Lake	n.d.	<1-20	n.d.	?	1	Overmann et al., 1996
Mariager Fjord	0.6	(<5)	n.d.	C	8/7	Ramsing et al., 1996
	17.9	5.2	1.1	C	8/6	this study
Rotsee	8.6	n.d.	n.d.	C	5	Kohler et al., 1984
Saanich Inlet	0.7	(<1)	(<0.1)	D	4	Jacobs & Emerson, 1982
Solar Lake	130	170	n.d.	C	5	Jørgensen et al. 1979
	260	155	6	C	8/6	Zopfi, 1998, unpubl.

[a)] Region where maximal concentrations were measured. C: Chemocline; D: Bottom water; [b)] 1: Voltametry, 2: RP-HPLC (DTNP derivatization), 3: Cyanolysis, 4: Colorimetry, 5: Iodometry, 6: RP-HPLC (Bimane derivatization) 7: Ion chromatography, 8: RP-HPLC; [c)] not detectable, detection limits are given in parentheses; [d)] not determined

conditions (Zhang and Millero, 1993b). Sulfate, thiosulfate and to a lesser extent sulfite remain the dominant oxidation products. Under anoxic conditions, however, sulfide is rapidly oxidized by MnO_2 and hydrous Fe(III)-oxides with S^0 as the main oxidation product (Burdige and Nealson, 1986; Yao and Millero 1993, 1996). In a chemocline, sulfide is oxidized at low oxygen concentration, but unfortunately no data are available on the influence of manganese and iron-oxides on the products of sulfide oxidation at very low oxygen.

Elemental sulfur also is an important product of biological sulfide oxidation. Phototrophic (Madigan, 1988) as well as chemolithotrophic microorganisms can produce zero-valent sulfur during sulfide oxidation which is then either excreted into the environment (Jannasch et al., 1991) or stored inside the cells (LaRivière and Schmidt, 1992). During our investigations in Mariager Fjord, phototrophic sulfide oxidizing bacteria were apparently of no importance for sulfide oxidation and sulfur production. This was indicated by a very low light intensity of 0.003% of surface irradiance and the absence of significant amounts of bacterial pigments. With the exception of the Black Sea (Overmann et al., 1992; Repeta et al., 1989), there are presently no reports of phototrophic communities developing at less than 0.02% surface light intensity (van Gemerden and Mas, 1995). Nevertheless, in all MPN tubes for sulfur reducing bacteria we observed the development of *Chlorobium* sp. This indicates that in and below the chemocline viable phototrophic sulfide oxidizers were indeed present although only in low numbers. We do not rule out that these organisms may play a role in sulfide oxidation during other periods of the year. In early spring, when the primary production in the upper mixed layer is low and the water is much more transparent, or in early summer when the chemocline may be located between 7 and 8 m (Fenchel et al., 1995), the conditions could be favorable for these organisms.

The chemolithotrophic sulfide oxidizing bacteria grow on a wide variety of sulfur compounds (Kelly, 1989). Under well-oxygenated conditions sulfide is completely oxidized to sulfate by most of these organisms. Some species, however, excrete partially oxidized sulfur compounds under oxygen limitation (Justin and Kelly, 1978). Chemostat studies with *Thiobacillus thioparus* T5 revealed that the pattern of products formed during sulfide oxidation was dependent on the degree of oxygen limitation (van den Ende and van Gemerden, 1993). As the steady state oxygen concentration decreased in their chemostat experiments, the oxidation products of sulfide changed from predominantly SO_4^{2-} and traces of S^0 to an end-product composition of 59% S^0, 27% SO_4^{2-} and 13% $S_2O_3^{2-}$ under O_2 limiting conditions (free sulfide detectable). In the Mariager Fjord chemocline the peak of S^0 at 13.7 m and the observation of maximal $^{14}CO_2$ incorporation indicates the location of maximum bacterial sulfide oxidation (Fig. 6). The increasing concentrations of thiosulfate and sulfite with depth are consistent with oxygen limitation of the chemolithoautotrophic community and are analogous to the chemostat study of *Th. thioparus T5*. However, so far all ecophysiological studies on the sulfur metabolism of sulfide oxidizing bacteria were performed with *Thiobacillus* sp., which

actually need not be a dominant organism in the chemocline of stratified water columns. Jannasch et al. (1991) isolated several strains of *Thiomicrospira* sp. from the Black Sea, but their sulfur metabolism is largely unknown, and ecophysiological studies are missing. Nevertheless, the results from the incubation experiment, where the potential rate of sulfide oxidation was determined, also supports the conclusion that chemolithotrophic sulfide oxidation is the principal process of sulfide oxidation operating in the Mariager Fjord chemocline under steady state conditions. The potential rate at 13.2 m depth was about 15 times faster than the calculated initial rate for purely chemical oxidation. This water sample was taken 50 cm above the depth of where the most intensive bacterial sulfide oxidation was indicated. Even higher potential rates could be expected from that depth, provided that O_2 is supplied. In the natural environment oxygen is continuously transported to the chemocline which sustains sulfide oxidation even at O_2 concentrations below the detection limit. As demonstrated by the bottle incubations, there was no detectable sulfide oxidation at 13.5 and 14 m where the O_2 supply was cut off although the presence of sulfur intermediates indicate sulfide oxidation at these depths (Fig. 4d).

Thiosulfate and sulfite could also be formed by the chemical reaction of sulfide with sedimenting manganese oxides (Luther et al., 1988; Yao and Millero, 1993), which is consistent with the apparent production horizon for Mn^{2+}. The sulfur intermediates in the water just below the chemocline could be used as electron acceptors by heterotrophic microorganisms. On the other hand, it has been shown that microorganisms can gain energy for growth by disproportionating these compounds to sulfate and sulfide (Bak and Cypionka, 1987; Thamdrup et al., 1993). The region just below the chemocline may provide a suitable habitat for these types of microorganisms. Whereas it has been shown that in anoxic sediments about 57% of the thiosulfate is disproportionated (Jørgensen, 1990), this process has not been quantified in water columns.

In contrast to some earlier studies, we did not observe increasing concentrations of thiosulfate and sulfite in the deep water (Millero, 1991b; Vairavamurthy and Mopper, 1990), although we found traces of these two compounds throughout the anoxic water column. However, we are not sure whether about 0.2 µM SO_3^{2-} and $S_2O_3^{2-}$ represent the *in situ* concentrations or whether traces of sulfite and thiosulfate were produced during the derivatization reaction. Kinetic considerations ($dSO_3^{2-} / dt = k [H_2S] [O_2] = 1.55\ M^{-1}\ min^{-1}$ [$600*10^{-6}$ M] [$254*10^{-6}$ M] = $2.4*10^{-7}$ M min^{-1}; Zhang and Millero, 1993b) suggest that under the worst (highly oxic) conditions about 0.24 µM SO_3^{2-} min^{-1} could be produced by the reaction of sulfide with oxygen. Since oxygen was carefully excluded, this calculation is certainly an overestimate, but it nevertheless gives some idea of how inappropriate sample handling can influence the results. Great care has to be taken in interpreting such low concentrations of intermediates at high background concentrations of sulfide.

Due to the very low saturation constants (K_s) for oxygen and sulfide of 1 µM or below (Kuenen and Bos, 1989, van den Ende and van Gemerden, 1993) in chemolithotrophic sulfur oxidizing bacteria, the sulfide oxidation rate in these organisms is over a wide range rather independent of the substrate concentrations. In chemostat cultures of *Th. thioparus* T5 the sulfide oxidation rate was constantly highest at steady state oxygen concentrations from 80 µM to <1 µM (van den Ende and van Gemerden, 1993). Thus, an oxygenation event due to chemocline perturbation should have little effect on the biological rate of sulfide oxidation. On the other hand, such an event greatly boosts chemical oxidation (Table 1) since kinetically the rate of sulfide oxidation is second order (Eq. 3) with first order dependencies of oxygen and sulfide respectively (Zhang and Millero, 1994). We estimated that the potential rate of chemical sulfide oxidation varied by more than one order of magnitude, depending on the conditions prevailing in the chemocline, which again were determined by physical parameters influencing the dynamics of the water column. Under low kinetic energy and steady-state conditions biological sulfide oxidation is clearly dominant in the Mariager Fjord chemocline, whereas perturbations of the chemocline (by shear, internal waves, eddies from wind energy dissipation) increase the portion of concurrent chemical sulfide oxidation.

These basic considerations of kinetics could also help to explain why many experiments have failed to show significant contribution of microorganisms to the direct oxidation of sulfide, and not just of the intermediates S^0 and $S_2O_3^{2-}$ (Millero, 1991a; Sorokin, 1970). Often these experiments were conducted under air saturating conditions that greatly stimulate chemical sulfide oxidation and may even inhibit the biological sulfide oxidation, since some sulfide oxidizing organisms are microaerophilic (La Rivière and Schmidt, 1992). In addition, the water for sulfide oxidation experiments often was not sampled at a depth where the most pronounced contribution of microorganisms could be expected.

4.4 Flux calculations and dark $^{14}CO_2$ uptake rates

A pronounced peak of dark $^{14}CO_2$ fixation has repeatedly been observed in the chemocline of stratified water columns and is usually assigned to chemolithotrophic processes such as nitrification or sulfide oxidation (Borsheim et al., 1985; Indrebø et al., 1979b; Jørgensen et al., 1991; Tuttle and Jannasch, 1979). In Mariager Fjord we determined an incorporation rate of 10.4 mmol $^{14}CO_2$ m^{-2} d^{-1} in the chemocline, where highest sulfide oxidation rates were indicated. We estimated the relative contribution of the different lithotrophic processes by combining the calculated fluxes (Table 2) with yield factors obtained from pure culture studies. Yield factors indicate how much CO_2 is fixed per oxidized substrate molecule and can vary according to growth conditions. For *Thiobacillus denitrificans*, for example, it has been shown in chemostats that the yield increased with decreasing steady state oxygen concentrations (Justin and Kelly, 1978). Yield factors and the calculated dark $^{14}CO_2$ rates for sulfide-, ammonium- and methane oxidation are summarized in Table 5. The fluxes of

reduced species are apparently not sufficient to account for the determined dark $^{14}CO_2$-uptake rate in the chemocline. This feature was also observed by Fenchel et al. (1995) who determined almost identical rates of dark $^{14}CO_2$ fixation. In other systems such as the Black Sea (Jørgensen et al., 1991) or Saelenvann estuary (calculated from values of Indrebø et al. 1979a, 1979b) dark $^{14}CO_2$ fixation rates nicely corresponded to the sulfide flux. This is clearly not the case for Mariager Fjord. Even calculations with maximum yield factors show that the lithotrophically driven $^{14}CO_2$ fixation accounts for only 37% of the observed dark fixation rate.

Table 5: Potential dark $^{14}CO_2$ fixation rate fueled by sulfide, ammonium, and methane oxidation, calculated under the assumption that the substrates are oxidized in the chemocline only.

Compound	Oxidation rate (mmol m^{-2} d^{-1})	Yield Factor* (mol CO_2 mol^{-1} Substr.)	$^{14}CO_2$ fixation rate (mmol m^{-2} d^{-1})
Sulfide	6.16	0.11 - 0.58 [a)]	0.67 - 3.57
Ammonium	1.95	0.03 - 0.11 [b)]	0.06 - 0.21
Methane	0.05**	0.10 - 0.14 [c)]	0.005 - 0.007
Total			0.74 - 3.79

* Reported minimal and maximal values are depicted; ** Methane oxidation rate determined by Fenchel et al. (1995); [a)] Jørgensen et al. (1991), Kelly (1989) [b)] Painter (1970), Belser (1984), Glover (1985); [c)] Higgins et al. (1981), Shishkina and Trotsenko (1985)

Possible reasons for this large discrepancy could be that sulfate reduction and, therefore, the fluxes of sulfide and ammonium were underestimated. Our flux calculations were constrained by the rate of sulfate reduction determined at one station, which of course may not be representative for the whole central basin. Sulfate reduction in basin wall sediments, for example, was not included in our calculations. Another factor possibly contributing to the electron donor fluxes could be sulfate reduction in the water column which has been found to take place in the chemocline and anoxic waters of some stratified water columns (Albert et al., 1995; Jørgensen et al., 1991; Smith and Oremland, 1987). In some cases, pelagic sulfate reduction was especially significant in or close to the chemocline (Indrebø et al., 1979b; Hastings and Emerson, 1988; Sorokin, 1970). Earlier attempts to quantify pelagic sulfate reduction in Mariager Fjord by means of $^{35}SO_4^{2-}$ radiotracer incubations and theoretical calculations did not indicate that sulfate reducing bacteria in the anoxic water body contribute significantly to the sulfide flux (Ramsing et al., 1996). Fenchel et al. (1995) noted that, below

25 m, sulfate reduction in the water column was indicated by a convex sulfide profile; however, we did not observe a similar feature in our profiles.

In addition to chemoautotrophic microorganisms, which use the enzyme ribulosebisphosphate-carboxylase (RuBisCO) to assimilate CO_2, heterotrophic bacteria can also contribute to the dark incorporation rate (Sorokin and Donato, 1975; Sorokin, 1970; Tuttle and Jannasch, 1979). CO_2 fixation in heterotrophic organisms is an anaplerotic reaction and serves only to regenerate metabolic intermediates that have been drained off from the tricarboxylic acid cycle for biosynthesis. The most important and most intensively studied enzyme in this context is phosphoenolpyruvate-carboxylase (PEPcase) which forms oxaloacetate by the addition of CO_2 to phosphoenolpyruvate (Overbeck and Sako, 1990). Enzyme activity measurements of PEPcase and RuBisCO in the chemocline of the Cariaco Trench revealed that CO_2 uptake by heterotrophic organisms can be equally important as autotrophic CO_2 fixation (Morris et al., 1985).

Many studies have shown that there is a relationship between the CO_2 uptake and the growth rate of heterotrophic bacteria (Overbeck, 1979; Overbeck and Sako, 1990). CO_2 is only taken up by heterotrophs in the presence of a suitable substrate for growth (i.e. biomass formation) and dark $^{14}CO_2$ uptake can therefore be viewed as a relative measure of the overall biosynthetic activity. Low molecular weight organic matter has been shown to act as activator for PEPcase activity (Overbeck, 1994). It should be noted that, under *in situ* conditions the dissolved organic substrate is in most cases a limiting factor for heterotrophic CO_2 uptake (Overbeck, 1979). However, in a chemocline where organic matter is trapped in a density gradient, perturbations of the chemocline may have a great influence on the overall heterotrophic activity since partially degraded organic material from below the chemocline is exposed to oxic conditions where it is readily metabolized. A strong increase in the activity of heterotrophic bacteria has been observed in mesocosm studies after inducing turbulence at the oxic-anoxic interface (Donner, 1997; Donner et al., 1996). We suggest that this was also coupled with an increase of the dark CO_2 fixation rate.

We do not have quantitative data to support our assumptions, but we emphasize that bacterial dark $^{14}CO_2$ incorporation rate is not necessarily only a measure for lithoautotrophic (i.e. nitrifying or sulfide oxidizing) biomass formation but also includes the uptake by heterotrophic microorganisms. This could be especially significant in Mariager Fjord where the productivity in the mixed layer is high and the chemocline shallow. Due to the high input of organic material to the chemocline the heterotrophic activity could mask the autotrophic $^{14}CO_2$ fixation signal.

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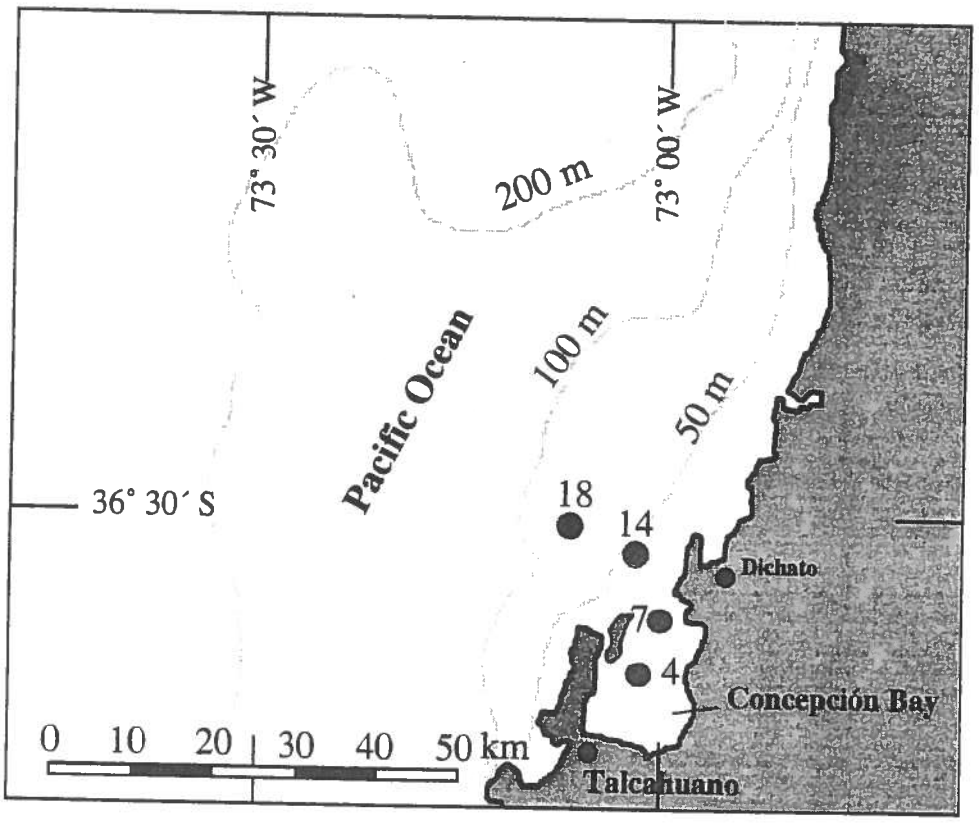

Figure 1: Sampling sites on the continental shelf and within the Bay of Concepción

CHAPTER 3

Nitrogen, Carbon and Sulphur Metabolism in Natural *Thioploca* Samples

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Abstract

Filamentous sulfur bacteria of the genus *Thioploca* occur as dense mats on the continental shelf off the coast of Chile and Peru. Since little is known about their nitrogen, sulfur, and carbon metabolism, this study was undertaken to investigate their (eco-)physiology. *Thioploca* is able to store internally high concentrations of sulfur globules and nitrate. It has been hypothesized earlier that these large vacuolated bacteria can oxidize sulfide by reducing their internal nitrate. We examined this nitrate reduction by incubation experiments of washed *Thioploca* sheaths with trichomes in combination with ^{15}N-compounds and mass spectrometry and found that these *Thioploca* samples produce ammonium at a rate of 1 nmol min^{-1} mg $protein^{-1}$. Controls showed no significant activity. Sulfate was shown to be the end product of sulfide oxidation and was observed at a rate of 2-3 nmol min^{-1} mg $protein^{-1}$. The ammonium and sulfate production rates were not influenced by sulfide addition, suggesting that sulfide is first oxidized to elemental sulfur and that in a second, independent step elemental sulfur is oxidized to sulfate. The average sulfide oxidation rate measured was 5 nmol min^{-1} mg $protein^{-1}$ and could be increased to 10.7 nmol min^{-1} mg $protein^{-1}$ after the trichomes were starved for 45 h. Incorporation of ^{14}C-labeled CO_2 was 0.4-0.8 nmol min^{-1} mg $protein^{-1}$, which is half the rate calculated from sulfide oxidation rates measured. 2-^{14}C-acetate incorporation was 0.4 nmol min^{-1} mg $protein^{-1}$, which is equal to the CO_2-fixation rate, and no $^{14}CO_2$ production was detected. These results suggest that *Thioploca* species are facultative chemolithoautotrophs capable of mixotrophic growth. Microautoradiography confirmed that *Thioploca* cells assimilated the majority of the radiocarbon from 2-^{14}C-acetate, with only a minor contribution by epibiontic bacteria present in the samples.

1. Introduction

Massive communities of *Thioploca* species occur as dense mats in the top sediment underlying the oxygen minimum zone of the continental shelf off the coast of Chile and Peru (Jørgensen and Gallardo, 1998). Reaching down to 5-10 cm into the sediment, the total biomass (including sheaths) of these colorless sulfur bacteria may be as high as 800 g wet weight m^{-2} (Schulz et al., 1996), potentially covering several thousands of square kilometers along 3000 km distance.

Thioploca chileae and *Thioploca araucae* are the two dominant species in the mat, measuring 12-22 and 28-42 µm in diameter, respectively (Schulz et al., 1996; Teske et al., 1995). Both species produce 2-7 cm long trichomes (filaments), each of which consists of a

uniseriate row of many vacuolated cells. Morphologically and phylogenetically, they are similar to vacuolated *Beggiatoa* species (Nelson et al., 1986) and it has been suggested that their physiology might be similar as well. A chief difference between the genera is, however, that *Thioploca* produces characteristic bundles of usually 10-20 trichomes, surrounded by 10-15 cm long sheaths of up to 1.5 mm in diameter. Individual trichomes can glide independently within the sheaths and reach out up to 3 cm into the water phase above the sediment (Huettel et al., 1996). In general, *Thioploca* species and *Beggiatoa* species appear to occupy different niches, the former living in vertical and horizontal sheaths down to 10 cm in sediments that contain relatively little sulfide. In contrast, *Beggiatoa* species live at or in the top layer of sediments that have relatively high sulfide concentrations.

Since their discovery by V. A. Gallardo (Gallardo, 1963; Gallardo, 1977) it has been assumed that the *Thioploca* mats play a crucial role in balancing the sulfur cycle of their marine habitat by reoxidizing all, or at least a substantial portion of the sulfide produced in the sediment. The sulfide results from high rates of bacterial sulfate reduction, up to 2.4 g S m^{-2} day^{-1} (Ferdelman et al., 1997), driven by extremely high primary productivity (up to 9.6 g C m^{-2} day^{-1}) over the continental shelf (Fossing et al., 1995). Recently, *Thioploca* spp. have been identified off the coast of Namibia (Gallardo et al., 1998), where similar oceanographic conditions exist, i.e. upwelling, high primary productivity and oxygen depleted bottom water.

Given that the high remineralization rates of organic compounds result in the often observed depletion of oxygen in the bottom water overlying the sea floor (Fossing et al., 1995; Gallardo, 1977; Schulz et al., 1996), the question arose as to which electron acceptor might be used for the reoxidation of all the sulfide produced in these sediments. When it was discovered that the vacuolated *Thioploca* species living in the mat were capable of accumulating up to 500 mM of nitrate from the overlying water (containing ~ 25 µM, Fossing et al. 1995), it was hypothesized that *Thioploca* species would be able to use the nitrate as terminal electron acceptor for sulfide oxidation (Fossing et al., 1995). It was assumed that nitrate would be reduced to dinitrogen, although no experimental data was available to support this (Fossing et al., 1995). The question was, therefore, still open as to whether dinitrogen gas or ammonium would be the product. This was particularly interesting in view of the finding by McHatton *et al.* (1996) that also vacuolated *Beggiatoa* species are capable of accumulating and reducing nitrate and in view of conflicting observations by others with respect to the final product of nitrate reduction by the non vacuolated *Beggiatoa alba*, i.e. ammonium or dinitrogen gas (Sweerts et al., 1990; Vargas and Strohl, 1985).

So far, it has not been possible to cultivate *Thioploca* species in pure culture. The same is true for the vacuolated (nitrate accumulating) *Beggiatoa* species. Hence, little is known about their (eco-)physiology, specifically their sulfide and sulfur oxidation rates, respiration of oxygen and/or nitrate, growth rate, capability to grow autotrophically, heterotrophically or

mixotrophically. Clearly, this knowledge is essential for understanding the role of *Thioploca* species in their habitat.

McHatton *et al.* (1996) studied partially purified cultures of naturally occurring populations of large vacuolated *Beggiatoa* species and showed that these organisms contain substantial activities of membrane-bound nitrate reductase, indicating that they may indeed be capable of using nitrate as the terminal electron acceptor. Ribulose-1,5-bisphosphate-carboxylase was also detected at significant activities, evidencing that the vacuolated marine *Beggiatoa* species are capable of autotrophic growth. Using rinsed samples of *Thioploca* material from a mat, Ferdelman *et al.* (1997) were able to demonstrate CO_2 fixing capacity in these preparations, indicating that *Thioploca* has an autotrophic potential.

Since *Thioploca* species live at high densities in the mats of the Chilean marine sediments and could be seen with the naked eye, we decided that it should be possible to obtain samples of these organisms, sufficiently pure to perform physiological experiments. We developed a simple method by which we handpicked individual sheaths with trichome bundles with forceps from the top 2 cm sediments incubated under a dinitrogen (N_2) atmosphere. After collecting and washing, cells were used for various experiments. Using (radio-) labeled and unlabeled substrates, a study was made of carbon, nitrogen and sulfur metabolism. The observed activities were compared with data obtained from field measurements. The results indicate that *Thioploca* species are (metabolically) highly active under anoxic conditions and that they can play a significant role in the total oxidation of sulfide in the mat under anoxic conditions in the presence of nitrate. They appear to be facultative chemolithoautotrophs with a mixotrophic potential, meaning they can use sulfide or sulfur as energy source for growth and CO_2 fixation and can use acetate under these conditions as an (additional) carbon source. Evidence presented in this study, points to ammonium as the end product of nitrate reduction, although conversion to dinitrogen gas cannot be ruled out. Oxygen at approximately 10% air saturation did not inhibit the observed CO_2 fixation.

2. **Material and Methods**

2.1 Sampling and Incubation

Samples were collected in January and February 1997 on the continental shelf within the Bay of Concepción, central Chile, on board the R/V *Kay Kay* and the laboratory work was performed at the Marine Biological Station of Dichato, both of the University of Concepción. Sampling was performed at 34 m water depth on Station 7 (30) at 36°36'5''S, 73°00'6''W. At this station, at the time of sampling, the percentage of *T. araucae* in the upper 2 cm of the sediment was 39-67 %. The ratio of the biovolumes of *T. araucae* and *T. chileae* were

approximately 70 : 30 and, therefore, the majority of the community consisted, in biovolume, of the species *T. araucae*.

Sediment samples were obtained by a Rumohr corer using Plexiglas cores (9.5 cm inside diameter), stored at 4°C and processed within 8 days after sampling. The top 1-2 cm was removed and placed on ice in an N_2-filled glove bag (Sigma-Aldrich). *Thioploca* sheaths with bundles of trichomes (of 1-2 cm in length) were collected with forceps and transferred to synthetic medium (containing per liter: 25 g NaCl, 6 g $MgSO_4$•7 H_2O, 1 g $CaCl_2$, 0.5 g K_2HPO_4, 0.1 g KH_2PO_4, 0.5 g $NaHCO_3$, pH 7) or to synthetic medium without $NaHCO_3$, supplemented with 0.05 % (w/v) thioglycolate and 1 mg l^{-1} catalase (Sigma-Aldrich, the Netherlands). The latter medium was found to give the best results and will be referred to in the text when using the term 'medium', unless stated otherwise. Media were sparged with dinitrogen (N_2) for 30 minutes for anoxic conditions and subsequently stored at 4°C. Short before use, media were sparged again with N_2 for 10 minutes, unless stated otherwise, while kept cold. Under a N_2 atmosphere in a glove bag, the sheaths with trichome bundles were washed twice, by transferring them into fresh medium, and incubated in airtight 6 ml vials, equipped with rubber septa (Exetainer, Labco, High Wycombe, UK). The medium in the vial was changed twice by decanting under a N_2 atmosphere, with minimal disturbance of the sheaths and trichome bundles. The incubation volume was adapted to the conditions of the experiment performed. Under anoxic conditions substrates were injected through the rubber septum. Residual sediment, left after washing *Thioploca* trichome bundles, was used as a control. In these controls, the amount of sediment used was 5 to 10 times higher than the estimated contamination of sediment attached to washed sheaths with trichome bundles. Another control consisted of disrupted *Thioploca* sheaths with trichome bundles (equal to the amount used in the experiment) which had been disintegrated mechanically by a Potter homogenizer (Fisher Scientific, the Netherlands). This control was necessary because observations under the fluorescence microscope had shown that sheaths were covered with epibiontic bacteria, including sulfate reducing filamentous bacteria of the genus *Desulfonema* (Teske and Widdel, unpubl.). After the Potter treatment, it was observed under the fluorescent microscope that *Thioploca* trichomes were mechanically disrupted, whilst the majority of the epibiontic bacteria remained intact. Bundles consisting of sheaths with bundles of trichomes will be referred to as "trichome bundles", while sheaths with bundles treated with a Potter will be referred to as "disrupted trichome bundles". The method of handpicking *Thioploca* sheaths with trichome bundles had a bias towards *Thioploca araucae*, which led to a majority, in biovolume, of this species in the samples (80-90%).

For each experiment, approximately 100 *Thioploca* trichome bundles were collected in a final volume of 3.5 ml under an N_2 atmosphere in gas tight vials, unless stated otherwise. Vials were incubated with substrates in a waterbath of approximately 12°C. At specific time-intervals,

samples were taken with a syringe, previously flushed with dinitrogen, and analyzed for ammonium, nitrite, sulfide, thiosulfate, and sulfate.

2.2 Analytical procedures

Nitrite and ammonium in the supernatant were determined colorimetrically as described by (Griess-Romijn van Eck, 1996) and (Fawcett and Scott, 1960), respectively. Intracellular nitrate concentrations were measured with a miniaturized version of the standard colorimetric method of Grasshoff et al. (1983). Nitrate was measured in 100 µl extracts of rinsed and dried *Thioploca* trichomes. Trichomes of 5-40 mm in length were dissected under the microscope with the help of forceps and needles. Length and width of these trichomes were measured and after washing and drying the filaments were resuspended in 50 µl of distilled water to measure the nitrate concentration. Biovolume was calculated from trichome length and width. An average nitrate concentration (n = 27) of 160 ± 150 mM was found. Protein was determined by the micro-biuret method of Goa (1953). The observed protein concentrations were in agreement with calculations for expected protein content. Where no protein measurements were available (in experiments where labeled compounds were used), a protein content of 315 ± 95 µg was assumed for 100 *Thioploca* sheaths with trichomes (which is based on an average of 34 protein measurements on *Thioploca* samples). Thiosulfate was derivatized with monobromobimane (Fahey and Newton, 1987) and analyzed by reversed-phase HPLC (Rethmeier et al., 1997). Sulfide was determined either colorimetrically according to Cline (1969), or by the method described for thiosulfate determination. Standards for sulfide and thiosulfate were prepared in degassed 'sulfate-free'-medium ($MgCl_2$ instead of $MgSO_4$ and without thioglycolate and catalase). Sulfate was determined by non-suppressed ion-chromatography as described by (Ferdelman et al., 1997). Since high concentrations of chloride interfere with sulfate analysis by ion-chromatography, chloride was removed from the samples by adding 40 mg Ag^+-loaded cation-exchanger (Biorad Ag 50W-X8) to 150 µl sample and incubated for 2 hours at room temperature. After centrifugation and filtering, the sample was analyzed. Standards were treated in the same way.

2.3 ^{15}N-experiments and mass spectrometry

Under anoxic conditions a concentrated solution of $Na^{15}NO_3$ was added to gas tight vials, each with 120 *Thioploca* bundles in 4.5 ml medium. No direct protein measurements could be performed and, therefore, a total protein content of 378 ± 114 µg was assumed on basis of the average protein content of 100 *Thioploca* bundles (see above). The headspace was changed with Helium (He) and at certain time intervals samples were taken for analysis. Total nitrite and ammonium concentrations were measured immediately after centrifugation. For determining the concentration of $^{15}NO_3^-$, $^{15}NO_2^-$, and $^{15}NH_4^+$, samples were removed with a

syringe, centrifuged, sterilized by passing the supernatant through a 0.2 µm filter (Dynagard, Microgon Inc., CA, USA), acidified to pH 4-5 and stored at -20°C until time of analysis. At the end of the experiment 1-2% formaldehyde (final concentration) was added to the vial and pressure was equilibrated with He. Vials were stored at 4°C until the headspace could be analyzed for ^{15}N-labeled N_2. Analysis and mass spectrometry were performed at the Institute of Biological Sciences, University of Aarhus. For determining concentrations and isotopic composition of $^{15}NO_3^-$ and $^{15}NO_2^-$, samples were neutralized and incubated with denitrifying bacteria to convert these compounds to N_2 for mass spectrometry analysis (Risgaard-Petersen, 1993). The labeling pattern of the obtained N_2 gives an indication of the ratio of labeled and unlabeled nitrate and nitrite present in the samples. However, the denitrifiers used can reduce both nitrate and nitrite. Therefore, the recovered N_2 is the product of reduction of nitrate as well as nitrite, present in the medium. Standards with known concentrations of $^{15}NO_3^-$ and $^{15}NO_2^-$ (120 µM) were included in the assay and confirmed that the conversion efficiency was consistent (SD = 4%) and that residual nitrate and nitrite concentrations were insignificant.

To analyze isotopic composition of the NH_4^+ formed, hypobromite was added for specific oxidation of ammonium to N_2 (Risgaard-Petersen et al., 1995). The mass spectrometer measured single and doubly labeled dinitrogen ($^{14,15}N_2$ and $^{15,15}N_2$) in excess of natural background. From this the recovery of added ^{15}N in the sampled N_2, NO_3^-, NO_2^-, and NH_4^+ was calculated. Dinitrogen is formed by random isotope pairing and, therefore, the ratio of labeled nitrogen recovered as $^{14,15}N_2$ versus the recovery as $^{15,15}N_2$ was used as a minimum estimate of the ^{14}N:^{15}N ratio in the source (Nielsen, 1992). If the source is isotopically uniform and constant, the estimate is correct. If several pools are involved, i.e. discrete intracellular nitrate pools in the incubation vial or nitrate and nitrite in the water samples, the true representation of unlabeled nitrogen cannot be much less (max. 0.5 nmol N less), but can be higher, depending on the pool sizes and isotopic variations.

2.4 ^{14}C-labeled $NaHCO_3$, 2-^{14}C- labeled acetate and ^{3}H-labeled acetate incorporation experiments

Several vials were incubated with approximately 30 *Thioploca* bundles in 1.3 ml medium, in the dark. On the basis of the average protein content of 100 *Thioploca* bundles (see above), the total protein for 30 bundles was assumed to be 94.5 ± 28.5 µg. 100 µM $NaNO_3$, 25 µl CO_2 gas (headspace was approximately 5 ml) and, for all experiments, 1 mM HCO_3^- was added to the medium. Under anaerobiosis, 0.005 nmol ^{3}H-labeled acetate (~500 µCi) or 0.034 µmol of a ^{14}C-labeled acetate solution (~5 µCi) was added in case of the labeled acetate experiments. For the labeled bicarbonate experiments, 0.139 µmol (~ 10 µCi) or 1.85 µmol of a

neutralized ^{14}C-labeled $NaHCO_3$ solution (~100 µCi, for microautoradiography experiments) was added. Experiments with ^{14}C-labeled bicarbonate were performed in the absence as well as in the presence of approximately 70 µM sulfide. At certain time intervals a vial was opened and the supernatant analyzed for NO_2^- and NH_4^+. The pellet of *Thioploca* bundles or debris from disrupted bundles were washed four times (by vigorous mixing and subsequent centrifugation), in medium with 10 mM acetate, when incubated with labeled acetate, or with 10 % TCA (trichloroacetic acid), when incubated with $NaH^{14}CO_3$, and then added to 2.4 ml H_2O and 7.5 ml scintillation liquid (EcoLite (+), ICN Biomedicals). This suspension was subsequently analyzed in a Scintillation Counter (Packard Liquid Scintillation Analyzer, Model 1600 TR). When necessary, CO_2 was trapped by suspending in the gas tight vial, a small cup filled with 100 µl 2 M NaOH. After opening of the vial this solution was neutralized and added to scintillation liquid and counted. Experiments with ^{14}C-labeled acetate took 3 hours, experiments using $NaH^{14}CO_3$ were incubated for 4 hours (to test the influence of oxygen, trichomes were incubated for 22 h) and trichomes used for microautoradiography were incubated for 4 h or 20-22 h.

2.5 Microautoradiography

Microautoradiography was performed on the experiments described above. Following incubation with ^3H-labeled acetate or with ^{14}C-$NaHCO_3$, bundles were washed 6 times with medium with 1 mM acetate or with 1 mM HCO_3^-, respectively. Individual sheaths were then sorted onto 25mm HA millipore filters (Millipore Co., Bedford, MA, USA) or left in 2 ml medium with 2% formaldehyde. Filters were subsequently washed in filter sterilized (first through 0.45 µm then through 0.2 µm, millipore GFF (Gelman) filters) medium with 1 mM phosphate buffer. After drying, the filters were stored at 4°C. At the end of the cruise the filters and samples, stored in 2% formaldehyde, were analyzed. Some filters were stained with 2% (w/v) erythrosin-B and subsequently destained by placing them face up on deionized H_2O-saturated pieces of gauze. Filters were air dried, attached to microscope slides and optically cleared by fuming acetone (Paerl, 1974). Cleared filters were prepared for microautoradiography by dipping them in Kodak NTB-2 Nuclear track emulsion. After exposure (1-3 weeks), autoradiographs were developed (Kodak D-19 developer), fixed, rinsed and air dried prior to microscopic examination with a Nikon Labophot 2 phase contrast microscope at 200-400X. Photographs were recorded on either Ilford Pan-F fine grain B & W or Kodachrome 200 color slide 35 mm films.

2.6 Calculations

^{15}N-experiments: the ratio between added ^{15}N-nitrate and intravacuolar ^{14}N-nitrate. Addition of 100 µM ^{15}N-labeled nitrate in 4.5 ml medium yields 0.45 µmol. 120 *Thioploca* bundles have a protein content of approximately 0.38 ± 0.11 mg (see analytical procedures). Assuming that 50% of the dry weight is protein, that 24% of the wet weight is dry weight and knowing that 90 % of the cell is vacuole (as measured in this study and by Maier et al., 1990), then the total wet weight of 120 bundles will be (0.38 x 2 x (100/24) x (100/10) =) 31.7 ± 9.17 mg, of which 28.5 ± 8.25 mg is vacuolar liquid. Assuming that 1 mg is equal to 1 µl of liquid in the vacuole, then the volume of all vacuoles in the bundles used for the experiments will be approximately 28.5 ± 8.25 µl. If all the added ^{15}N-labeled nitrate is transported into the vacuoles, then this would lead to a concentration of the label of 15.8 ± 4.57 mM (0.45 µmol in 28.5 µl). Since the vacuoles are filled with an average of 160 mM (see Materials and Methods) unlabeled nitrate, the labeled nitrate will be diluted to 9.9 ± 2.85 %. If *Thioploca* trichome bundles would be damaged and all internal nitrate would be released, then approximately 5.6 µmol (160 mM in 28.5 µl) would be released in 4.5 ml medium. This would lead to an increase in nitrate concentration of 1.2 mM, i.e. a 12-fold increase, which would be visible during measurement of ^{15}N:^{14}N ratio of the external nitrate pool.

Sulfide oxidation: the ratio between observed sulfide reoxidation rates and specific activity of *Thioploca*. Sulfate reduction rates measured in sediments at Station 7 at the time of sampling were approximately 30 mmol m^{-2} day^{-1} (26-37 mmol m^{-2} day^{-1}, Schulz et al., 1996). If all sulfide produced from this reduction was subsequently oxidized by the *Thioploca* mats, then the mats should be able to oxidize sulfide at the same rate, which is equal to 20.8 µmol m^{-2} min^{-1}. Schulz et al. (1996) estimated the wet biomass of trichomes without sheaths to be 50-120 g m^{-2}. Assuming an average of 85 g wet weight m^{-2} and knowing that 90% of the biovolume is taken up by the central vacuole, the active cytoplasm weighs approximately 8.5 g wet weight m^{-2}. This active cytoplasm is then responsible for the sulfide oxidation rate as stated above, which would give a specific rate of (20.8 / 8.5 =) 2.4 µmol min^{-1} g wet $weight^{-1}$. Assuming that 24% of wet weight is dry weight and that 50% of dry weight is protein, the sulfide oxidation rate *in vivo* should be (2.4 x (100/24) x 2 =) 20.4 nmol min^{-1} mg $protein^{-1}$. In analogy Ferdelman et al. (1997) found an *in vivo* sulfate reduction rate of approximately 17.5 mmol m^{-2} day^{-1} for Station 7. This reduction rate corresponds to a sulfide oxidation rate by *Thioploca* of 12 µmol m^{-2} min^{-1}. Making the same assumptions as above, this oxidation rate is equal to 11.8 nmol min^{-1} mg $protein^{-1}$.

3. Results

3.1 Cultivation and survival

Thioploca bundles, consisting of 10-20 trichomes in a sheath, were collected from the top 2 cm of the sediment with forceps and cleaned by several transfers through medium. For development of the method there were three parameters to be considered. Firstly, motility of *Thioploca* trichomes under a microscope was a measure of viability (Maier and Gallardo, 1984). Secondly, it was observed during the initial experiments that high nitrite (NO_2^-) concentrations (50-100 μM), in addition to high nitrate concentrations, were obtained within an hour after anoxic incubation of the bundles, suggesting lysis of the cells. Thirdly, from previous studies of *Thioploca* and large *Beggiatoa* species it was observed that they are highly sensitive to oxygen (Huettel et al., 1996; Møller et al., 1985) and that catalase is required in the growth medium of *Beggiatoa* species (Burton and Morita, 1964). These considerations led to several improvements of the final cleaning procedure. To avoid contact with oxygen all the different steps of the method (collection, washing, and incubation) were performed under a dinitrogen atmosphere. Sparging of the medium during incubation for anoxic conditions was avoided, because this mechanically affected the trichomes. To keep the growth medium anoxic thioglycolate was added as a reducing agent and also catalase was included in the synthetic medium. The endogenous ammonium production rate did not increase after including thioglycolate in the medium, suggesting that this compound was not used as a carbon source. Survival experiments as monitored under the microscope showed that the medium highly improved survival and that trichomes did not show a decrease in motility over two days of incubation. From similar survival experiments, it was further concluded that *Thioploca* cells could get damaged when transferred through a liquid-gas interface. To avoid this damage, washing was performed twice by draining off approximately 80 % of the medium, such that the trichomes were still in the liquid and then fresh medium was added.

Further improvement of the method was obtained by keeping the sediment on ice during collection and washing of the trichomes and by omitting bicarbonate to the medium, since removal of CO_2 during sparging of the medium caused an increase in the pH. In all subsequent experiments these cleaned *Thioploca* trichomes were used.

3.2 N-metabolism

Inside *Thioploca* cells, intravacuolar nitrate concentrations were measured up to 500 mM with an average of 160 ± 150 mM (n = 27). *Thioploca* trichome bundles incubated in medium without addition of NO_3^-, produced NO_2^- and NH_4^+. The average NH_4^+ production (with internal sulfur available, but no added external electron donor) of 8 independent measurements was 1.0 ± 0.3 nmol min^{-1} mg $protein^{-1}$, whereas NH_4^+ production by the

controls (disrupted trichome bundles or NO_3^--supplemented sediment) was 0.07 ± 0.03 nmol min^{-1} mg $protein^{-1}$ and 0.03 ± 0.01 nmol min^{-1} mg $protein^{-1}$, respectively. Nitrite production was negligible (<0.1 nmol min^{-1} mg $protein^{-1}$) in most experiments performed, but was sometimes observed at a maximum production rate of 1 nmol min^{-1} mg $protein^{-1}$.

^{15}N-labeling experiments. To determine whether *Thioploca* reduces NO_3^- to NH_4^+ or to N_2, experiments were performed using ^{15}N-labeled NO_3^-. After addition of ^{15}N-labeled NO_3^-, total NO_2^- and total NH_4^+, as well as ^{15}N-labeled NO_3^-/NO_2^- and NH_4^+ were followed over time (the ^{15}N-method does not differentiate between labeled NO_3^- or NO_2^-, see Materials and Methods). At the end of the experiment, total ^{15}N-labeled N_2 and the ratio between unlabeled and (singly or doubly) labeled N_2, were determined. Figure 1A shows that 95 % of the externally available NO_3^- or NO_2^- originated from the supplied ^{15}N-labeled NO_3^-. During the course of the experiment, the specific labeling of the extracellular nitrate pool remained 95% and did not decrease, indicating that the trichomes were not damaged and did not release $^{14}NO_3^-$ (see calculations in Materials and Methods). Figure 1A shows that nearly all of the nitrate was taken up linearly during the course of the experiment in approximately 3.5 hours. As calculated in Materials and Methods, if all of the ^{15}N-labeled NO_3^- were taken up by *Thioploca* trichomes, the label would be diluted inside the vacuoles to 9.9 ± 2.85 % (see calculations in Materials and Methods). The NH_4^+ produced during the experiment (1.8 ± 0.4 nmol min^{-1} mg $protein^{-1}$) was 44 % (at time 85 min) and 48 % (time 145 and 215 min) labeled (Figure 1B). This difference in specific labeling between the externally available nitrate pool and the NH_4^+ produced indicates that the internal nitrate of *Thioploca* trichomes contributes substantially to the total NH_4^+ production. However, the amount of label is not diluted as much as would be expected if the entire labeled nitrate would first be taken up in the vacuole and subsequently reduced. Therefore, it seems that in the cytoplasm the ^{15}N-labeled nitrate is readily reduced before it reaches the vacuole. Figure 1C shows that at the end of the experiment N_2 had also been produced, but the amount was only 15% (nmol N/nmol N) of the total amount of nitrogen compounds produced. The specific labeling of the N_2 was substantially higher than that of NH_4^+, suggesting that epibionts might be responsible for this production, although the amount of unlabeled N_2 is a minimum estimate (see Materials and Methods). This implies that although N_2 appeared not to be a major product of the washed *Thioploca* sample, the present data cannot completely rule out that *Thioploca* can reduce nitrate to N_2, i.e. denitrify, in addition to the observed full reduction of nitrate to ammonium.

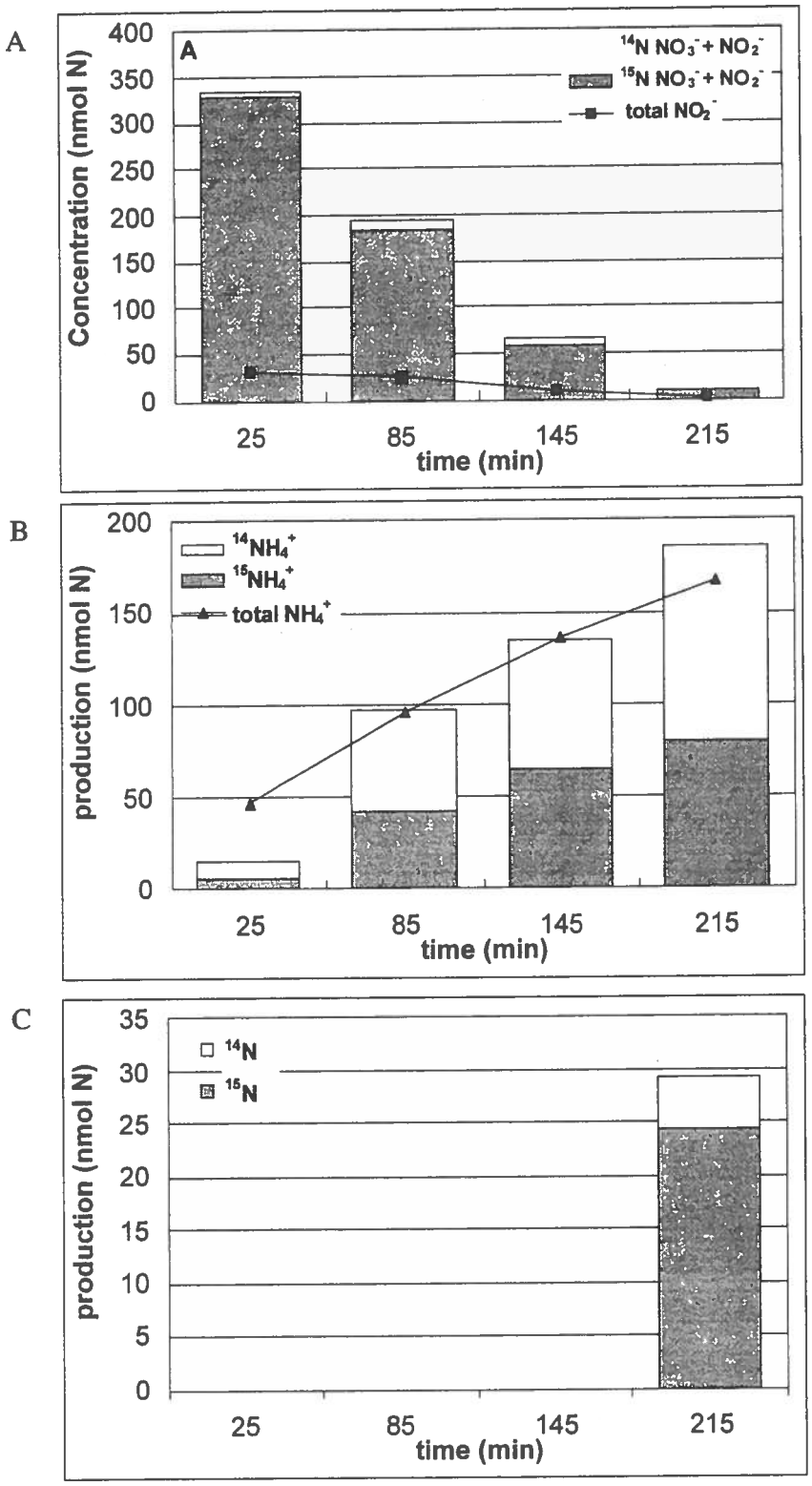

Figure 1: Distribution of label during ammonium and dinitrogen production by *Thioploca* trichome bundles after incubation in medium with ^{15}N-labeled nitrate under a Helium headspace. A; ^{14}N- and ^{15}N-labeled nitrate and nitrite in the growth medium, B; ^{14}N- and ^{15}N-labeled ammonium in the growth medium, C; total amount of ^{14}N- and ^{15}N-label derived from dinitrogen species in the headspace ($^{14,\ 14}N_2$, $^{14,\ 15}N_2$, $^{15,\ 14}N_2$, $^{15,\ 15}N_2$).

3.3 S-metabolism

To measure sulfide oxidation rates, *Thioploca* trichomes were incubated in medium. After addition of approximately 50 µM sulfide to the vials, sulfide, nitrite, and ammonium concentrations were followed with time. An ammonium production rate by the *Thioploca* suspension of approximately 1.9 nmol min^{-1} mg $protein^{-1}$ was observed, whereas the controls (sediment samples and samples treated in a Potter homogenizer) showed an activity of 0.02 and 0.04 nmol NH_4^+ min^{-1} mg $protein^{-1}$, respectively (Table 1). The sulfide consumption rate decreased with decreasing sulfide concentrations, but the average maximum rate was approximately 4.2 nmol min^{-1} mg $protein^{-1}$, whilst the controls showed a 10 to 200 -fold lower consumption rate. Since the control, which contained sediment, represented an overestimate of the amount of sediment attached to the trichomes, the contribution of the sediment to the total activity in live *Thioploca* experiments was negligible. Trichomes, which had been collected and incubated two days before the experiment was performed, showed continued motility under the microscope and an ammonium production rate of 3.2 nmol min^{-1} mg $protein^{-1}$ and a sulfide consumption rate of 5.5 nmol min^{-1} mg $protein^{-1}$, after addition of sulfide to the incubation medium. The cells appear to reduce their metabolic activity when no external substrate is present (NH_4^+ production rate is approximately 1 nmol min^{-1} mg $protein^{-1}$), but are able to respond quickly when substrate is encountered again (NH_4^+ production increases to 3.2 nmol min^{-1} mg $protein^{-1}$). An internal nitrate concentration of 160 mM would be sufficient for approximately 200 hours (given our estimate that 90% of the cell is vacuole and that 1 mg vacuolar liquid is 1 µl), with an NH_4^+ production rate of ± 1 nmol min^{-1} mg $protein^{-1}$. This experiment indicates that trichomes are still active and motile after two days and that the internal NO_3^- is sufficient for at least two days of normal metabolism without external supply of fresh substrate. In an experiment where two different concentrations of sulfide were added to *Thioploca* suspensions (Figure 2), a small accumulation of thiosulfate was observed, which was higher when the sulfide concentration was higher. This thiosulfate accumulation suggested that this compound may be a by-product or an intermediate in sulfide oxidation and, therefore, *Thioploca* might be able to oxidize thiosulfate to sulfate. To investigate this possibility, approximately 100 µM thiosulfate was added to *Thioploca* trichome bundles incubated in 'sulfate-free'-medium with $MgCl_2$ instead of $MgSO_4$ and without thioglycolate and catalase, to avoid interference with analytical measurements. In these experiments (results not shown) only a slight thiosulfate consumption was observed.

Table 1: Specific rates of ammonium production and sulfide consumption by *Thioploca* trichome bundles incubated in medium.

Sample	NH_4^+ production rate (nmol min^{-1} mg $protein^{-1}$)	HS^- consumption rate (nmol min^{-1} mg $protein^{-1}$)
Thioploca sheaths with trichomes	1.9	4.2
Thioploca sheaths with trichomes *	3.2	5.5
disrupted *Thioploca* sheaths with trichomes	0.04	< 0.02
Sediment (with 100 µM NO_3^-)	0.02	0.5

n.d.: not detectable; *: two-day old culture

Freshly harvested cells contain high concentrations of elemental sulfur (200 nmol mm^{-3}) and nitrate (160 mM), which could influence the observed oxidation rates. In line with the calculations made above for consumption of internal nitrate during starvation, it can be calculated that the internal sulfur would be sufficient for approximately 170 hrs. Thus, it was possible that (partially) starved cells would show higher oxidation rates. Therefore, suspensions of 50 *Thioploca* trichome bundles were S^o-starved for 45 hours by incubation in 4.5 ml 'sulfate-free'-medium in the presence of 50 µM nitrate. After 45 hours, approximately 135 µM sulfide or 70 µM thiosulfate was added to the suspensions and ammonium, sulfide, thiosulfate and sulfate concentrations were followed over time (results not shown). During anoxic S^o-starvation, NH_4^+ and SO_4^{2-} production by *Thioploca* trichome bundles was approximately 1 and 2-3 nmol min^{-1} mg $protein^{-1}$, respectively. In the control with disrupted trichome bundles, NH_4^+ and SO_4^{2-} production were 0.1 and 0.45 nmol min^{-1} mg $protein^{-1}$, respectively. This activity was ten times lower than the activity of the trichome bundles, indicating that epibiontic bacteria are not responsible for the observed sulfate production. Subsequent sulfide addition (135 µM) to these S^o-starved trichome bundles led to an initial sulfide consumption rate of 10.7 nmol min^{-1} mg $protein^{-1}$, which was the highest oxidation rate observed, whilst ammonium and sulfate production did not increase significantly. There was no production of sulfite during these experiments, however, accumulation of thiosulfate was observed in the disrupted control (up to 21 µM) during starvation. This control contained sulfur compounds released from the ruptured *Thioploca* trichome bundles, suggesting that epibiontic bacteria may be responsible for thiosulfate accumulation. After addition of sulfide to intact starved trichome bundles a thiosulfate accumulation occurred also (up to 10 µM). After addition of thiosulfate to S^o-starved

Thioploca trichome bundles, only a low thiosulfate consumption rate was measured, which was equal to the rate observed previously. Measurements of this consumption rate also showed high variability. Addition of thiosulfate had no effect on ammonium or sulfate production.

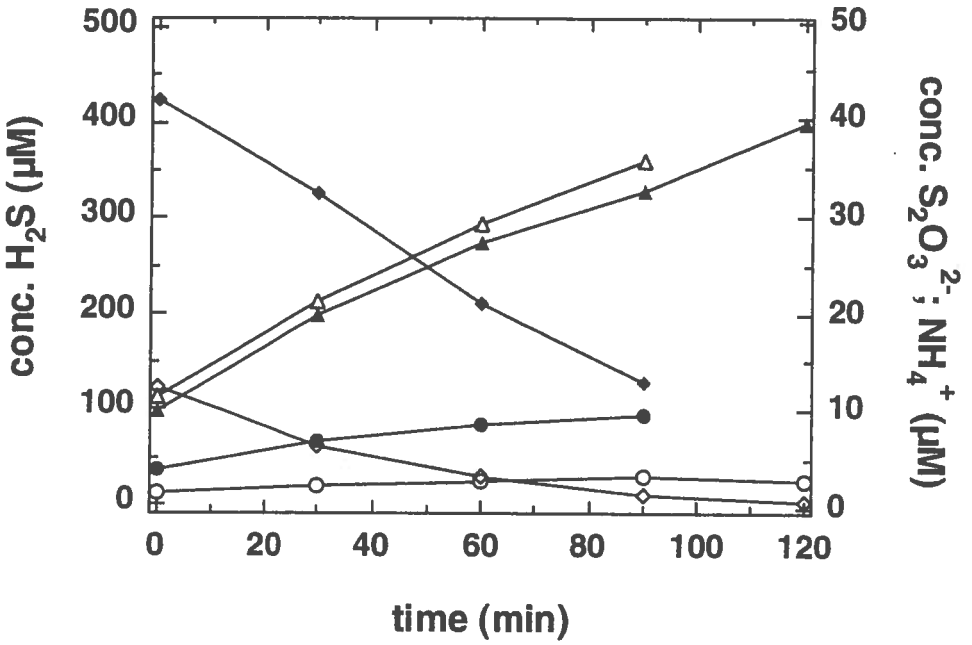

Figure 2: Thiosulfate production by *Thioploca* trichome bundles incubated anoxically in medium with two different initial sulfide concentrations. Open symbols; 100 µM initial sulfide concentration, closed symbols; 400 µM initial sulfide concentration. Symbols: triangle, ammonium; circle, thiosulfate; diamond, sulfide

3.4 C-metabolism

In order to gain some insight into the carbon source used by *Thioploca* for its cell material, experiments were performed with radioactively labeled acetate and bicarbonate additions (Table 2) in combination with microautoradiography.

^{14}C-labeled $NaHCO_3$. Addition of ^{14}C-labeled $NaHCO_3$ to a *Thioploca* suspension resulted in a linear incorporation rate of 0.4-0.8 nmol min^{-1} mg $protein^{-1}$ and an NH_4^+ production rate of approximately 1.3-1.7 nmol min^{-1} mg $protein^{-1}$. Addition of sulfide (ca. 70 µM) did not have a significant effect on the incorporation rate. The presence of low concentrations of oxygen (ca. 10 % air saturation) also did not have a significant effect on the rates of ^{14}C-labeled $NaHCO_3$ fixation. Control experiments with disrupted trichome bundles showed ^{14}C-fixation rates of only 0.01 nmol min^{-1} mg $protein^{-1}$, which is 1-3% of the rates observed in intact *Thioploca* suspensions.

Intact bundles obtained from these experiments were used for microautoradiography. Uptake of ^{14}C-label appeared to be largely dominated by *Thioploca* trichome bundles, since there was no significant uptake of label in epibiontic microbial cells associated with the sheaths. Microautoradiography and control experiments using disrupted trichome bundles, both show that the measured uptake rate of ^{14}C-label by the *Thioploca* suspension primarily represents the activity of the trichome bundles and not of epibionts. Differences in intensity of labeling among trichomes were observed, but no differences were observed that could be due to possible differences in the physiology of the two major species in the sample (*T. araucae* and *T. chileae*). Among individual trichomes labeling was homogeneously distributed along their entire length, and labeling was concentrated along the transverse walls (Figure 3), suggesting the presence of a vacuole.

Table 2: Specific rates of NH_4^+-production and ^{14}C-incorporation by *Thioploca* trichome bundles incubated in medium after addition of ^{14}C-labeled acetate or $NaHCO_3$.

Sample	NH_4^+ production rate (nmol min^{-1} mg $protein^{-1}$)	^{14}C-incorporation rate (nmol min^{-1} mg $protein^{-1}$
Th. trichomes + ~ 5 µCi ^{14}C-acetate	4.0	0.37
Th. trichomes + ~ 10 µCi $NaH^{14}CO_3$	1.3	0.5-0.8
Th. trichomes + ~ 10 µCi $NaH^{14}CO_3$ + 70 µM Na_2S	1.7	0.3-0.5
Th. trichomes + ~ 100 µCi $NaH^{14}CO_3$	1.2	0.25-0.35
Th. trichomes + ~ 100 µCi $NaH^{14}CO_3$ + 2 % O_2	0.65	0.36-0.48
disrupted trichomes + ~ 3 µCi ^{14}C-acetate	< 0.01	< 0.01
disrupted trichomes + ~ 100 µCi $NaH^{14}CO_3$	< 0.01	0.01

^{14}C- and ^{3}H-labeled acetate. After addition of ^{14}C-labeled acetate to *Thioploca* trichome bundles, an acetate uptake rate of approximately 0.4 nmol min^{-1} mg $protein^{-1}$ was observed and an NH_4^+ production rate of 4 nmol min^{-1} mg $protein^{-1}$. Unaccounted loss of label was less than 10%. ^{14}C-labeled CO_2 production was not significant (less than 2% of acetate incorporation), indicating that under these conditions (in the presence of internal sulfur) acetate was not used as a significant energy source. Control experiments with disrupted trichome bundles showed an incorporation rate of less than 0.01 nmol min^{-1} mg $protein^{-1}$, which was less than 2 % of the activity of intact *Thioploca* trichome bundles.

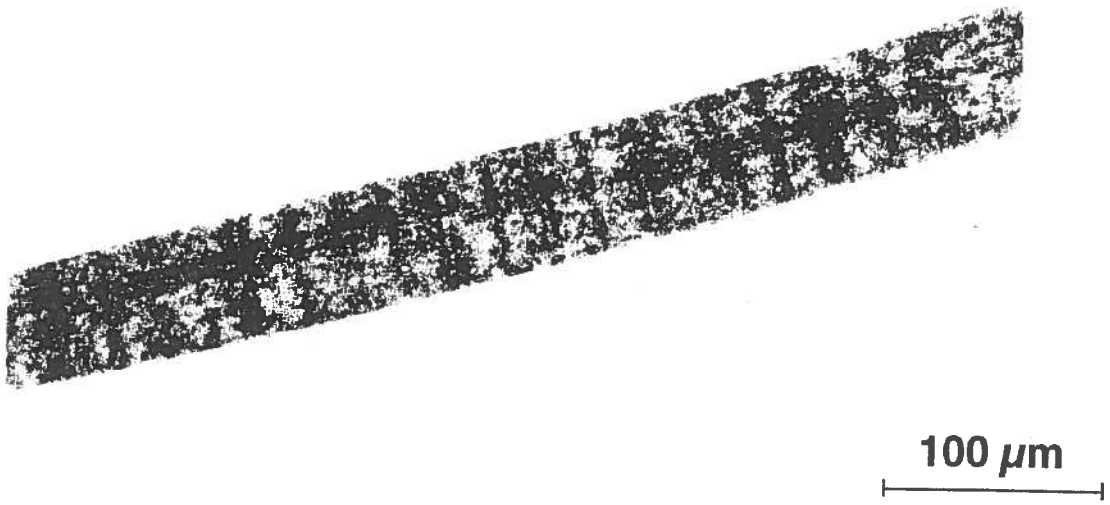

Figure 3: High magnification microautoradiogram of a single *Thioploca* trichome incubated with ^{14}C-labeled $NaHCO_3$.

Bundles incubated with ^{3}H-labeled acetate and showing similar activities, as described above, were examined by microautoradiography. Results indicate acetate uptake by trichomes as well as by bacteria associated with the sheath (Fig. 4). However, taking into account the volume-ratio between trichomes and attached bacteria, uptake of label was largely dominated by *Thioploca* trichomes. This indicated that measured uptake rates of label were primarily due to *Thioploca* trichomes. No differences were observed between the two species of *Thioploca* present. Labeling with the soft ß-emitter ^{3}H gives a higher resolution than ^{14}C and, therefore, shows more clearly the difference between uptake of label by epibionts and by *Thioploca* trichomes. Figure 5 shows, more clearly than with ^{14}C-label, that the ^{3}H-label is situated along the transverse cell walls. This reflects the presence of a large central vacuole, leaving the cytoplasm concentrated along the cell walls. The results with ^{3}H-labeling showed uniformity in cell to cell labeling along the entire length of a trichome, as described above for ^{14}C-labeling, as well as differences in cellular labeling between individual trichomes. Uniform trichome labeling was observed immediately after addition of the labeled acetate and increased in intensity with time, indicating accumulation of label, reflecting measured uptake rates.

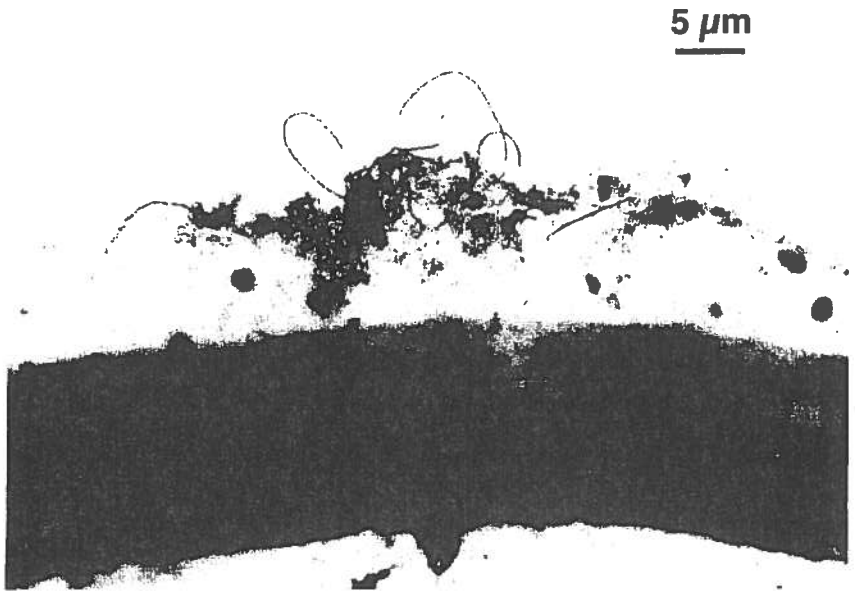

Figure 4: Low magnification microautoradiogram of a *Thioploca* sheath with trichomes incubated with ^{3}H-labeled acetate, showing ^{3}H-uptake by trichomes and associated bacteria. The heavily labeled trichomes are out of focus to show uptake by bacteria situated on the sheath. This figure has been selected for its high concentration of epibionts and is not representative for the overall results from the microautoradiography.

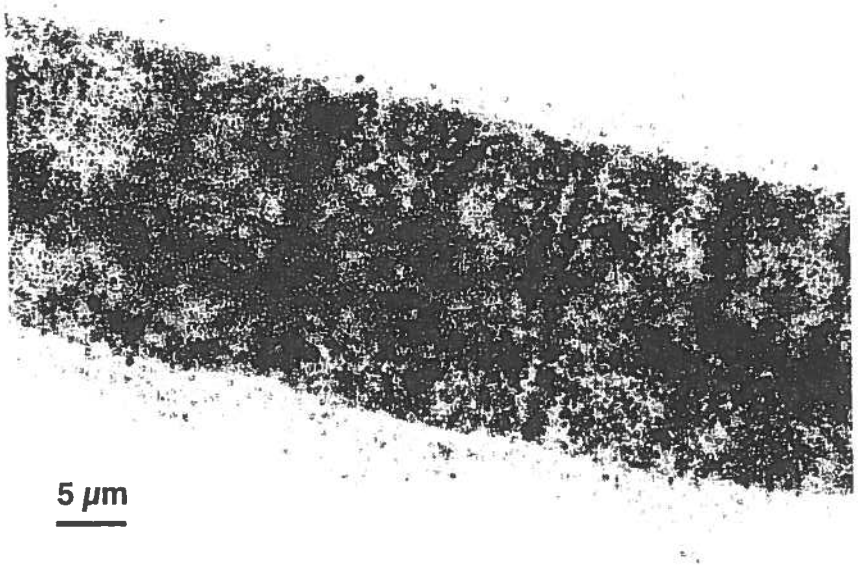

Figure 5: High magnification microautoradiogram of *Thioploca* trichome incubated with ^{3}H-labeled acetate.

4. Discussion

Filamentous sulfur bacteria of the genus *Thioploca* occur along the continental shelf off the coast of Chile and Peru. High sulfate reduction rates in *Thioploca* mats have been reported (Ferdelman et al., 1997). *Thioploca* species are able to store internally high concentrations of sulfur globules and nitrate. It is assumed that these vacuolated *Thioploca* species use their internally stored nitrate as a terminal electron acceptor for sulfide and sulfur oxidation (Fossing et al., 1995). The product of nitrate reduction, however, was still unknown. Also, *Thioploca* trichome bundles have been shown to take up both CO_2 and acetate, but quantitative data are lacking (Maier and Gallardo, 1984). Therefore, this study was undertaken to investigate carbon, nitrogen and sulfur metabolism in *Thioploca* species.

A method was developed to collect and clean individual sheaths with bundles of trichome bundles from the top 2 cm of the sediment. After collection and washing under a N_2 atmosphere, trichomes were still motile and could be used for physiological experiments. In the early stages of development of the method, high cellular or extracellular nitrite and nitrate concentrations were observed, possibly as a result of lysis of the cells. However, after adjustments (anoxic conditions, medium supplemented with thioglycolate and catalase, low temperature and avoiding transfer through gas-liquid interface) these nitrite and nitrate accumulations were no longer observed. *Thioploca* trichome bundles incubated for two days still showed activity comparable to activities measured immediately after incubation (Table 1), indicating that trichome bundles were able to survive and remain physiologically intact in the synthetic medium.

4.1 N-metabolism

During experiments performed with intact *Thioploca* trichome bundles, without addition of external substrate, an ammonium production rate of approximately 1 nmol min^{-1} mg $protein^{-1}$ was observed. Since in these experiments the only available substrates were internally stored sulfur and nitrate in *Thioploca* trichome bundles, it is highly unlikely that epibiontic bacteria were responsible for this NH_4^+ production. Experiments using ^{15}N-labeled nitrate resulted in uptake of all the labeled nitrate in approximately 3.5 hours. The specific label of the external NO_3^- pool remained 95% and was, therefore, not diluted during the course of the experiment, indicating that trichome bundles were not damaged and leaking out NO_3^-. Analysis showed an increase in NH_4^+ production (1.8 ± 0.4 nmol min^{-1} mg $protein^{-1}$) immediately after addition of labeled NO_3^-. The specific label of the NH_4^+ produced was maximally 48 %. This indicates that NH_4^+ is produced from a different NO_3^- pool than the external pool, since the external pool was more heavily labeled. The only other source of NO_3^- is the internal NO_3^- of *Thioploca* trichome

bundles, which is not available to epibiontic bacteria. This indicates that *Thioploca* species reduce NO_3^- to NH_4^+. Another argument is that there was no electron donor for NO_3^- reduction available in these experiments, except the internally stored sulfur.

If all the NO_3^- were taken up by *Thioploca* trichome bundles this would lead to an increase in NO_3^- within the vacuole of 15.8 ± 4.57 mM (see calculations in Materials and Methods). Since the average NO_3^- concentration in the vacuoles was found to be 160 mM, this would correspond to a dilution of the $^{15}NO_3^-$ to a specific labeling ($^{15}NO_3^-$: $^{14}NO_3^-$) of 9.9 ± 2.85 % (see calculations in Materials and Methods). If this NO_3^- pool would subsequently be reduced, then labeling of the NH_4^+ should be much lower than 48 %. The fact that the produced NH_4^+ is more heavily labeled suggests that during transport of the labeled NO_3^- across the membrane into the thin layer of the cytoplasm, it be readily reduced. If the transport rate of nitrate from the vacuole into the cytoplasm were in the same order of magnitude, then this would explain that the actual specific labeling of the cytoplasm is near 48 %.

N_2 was also detected in the headspace and was more heavily labeled than the NH_4^+ produced. However, since the amount of unlabeled N_2 was a minimum estimate (see Materials and Methods), the specific labeling of the produced N_2 can actually be lower than shown in Figure 1C, suggesting that the produced N_2 may also have a different specific labeling than the external pool of NO_3^-. Hence, on the basis of these data, one cannot completely exclude the possibility that *Thioploca* can also reduce NO_3^- to N_2. The amount of N_2 produced, however, was approximately 15% of the amount of NH_4^+ produced, emphasizing that under the conditions tested reduction of NO_3^- to NH_4^+ is the preferred pathway in *Thioploca*, and is probably used for energy conservation. Conservation of energy from NO_3^- reduction to NH_4^+ has also been found in *Sulfurospirillum deleyianum*, which uses sulfide as electron donor (Eisenmann et al., 1995), and in *Campylobacter* species (Stouthamer, 1988), where H_2 was used as an electron donor. The ecological implications of the finding that *Thioploca* prefers to produce NH_4^+ are significant, since this means that nitrate reduction by *Thioploca* does not lead to nitrogen loss in this vast ecosystem along the entire coast of Chile and Peru.

4.2 S-metabolism

After addition of sulfide to *Thioploca* trichome bundles, in a particular experiment, a sulfide oxidation rate of approximately 4.2 nmol min^{-1} mg $protein^{-1}$ was observed in the absence of external nitrate. The NH_4^+ production was 1.9 nmol min^{-1} mg $protein^{-1}$, resulting in a ratio of 2.2 between sulfide oxidized and NH_4^+ produced. If the sulfide were oxidized to elemental

sulfur and NO_3^- reduced to NH_4^+, then an expected ratio of sulfide to ammonium would be 4. If sulfide were oxidized to sulfate then a ratio of 1 is expected. The observed ratio suggests that the sulfide be oxidized to both sulfur and sulfate, since there was no significant accumulation of other (intermediate) sulfur species (i.e. sulfite and thiosulfate). Analogous to observations in marine *Beggiatoa* (Nelson et al., 1986), it is likely that the immediate product of sulfide oxidation is elemental sulfur, which is stored in *Thioploca* as globules. The elemental sulfur is then oxidized to SO_4^{2-} in a second, independent step, as suggested by Fossing *et al.* (1995). In experiments without addition of sulfide, sulfate production was observed at a rate of 2-3 nmol min^{-1} mg $protein^{-1}$, which must have originated from internal elemental sulfur. In the presence of sulfide, the SO_4^{2-} production rate did not increase significantly, suggesting that sulfide is oxidized to sulfur and that further oxidation of sulfur to SO_4^{2-} occurs independently of the presence of sulfide. In these two experiments, the ratio between SO_4^{2-} and NH_4^+ produced was approximately 1.5 in the absence and approximately 1.7 in the presence of sulfide. If NO_3^- is reduced to NH_4^+ and sulfur oxidized to SO_4^{2-}, then a ratio of 1.3 is expected. This is in agreement with the observed ratio in the absence of sulfide, indicating, again, that *Thioploca* trichome bundles reduce most NO_3^- to NH_4^+ under the conditions tested. It was also observed that addition of different concentrations of sulfide (100 µM and 400 µM) did not result in a significant increase in NH_4^+ production (Fig 2). This reconfirms that oxidation of sulfide, and subsequently sulfur, occurs independently. The observed ratios indicate that net sulfur accumulation will occur when external sulfide is present.

Addition of sulfide led to a small accumulation of thiosulfate ($S_2O_3^{2-}$) in the medium, suggesting that $S_2O_3^{2-}$ may be an intermediate in sulfur oxidation to sulfate. However, addition of $S_2O_3^{2-}$ to trichome bundles showed only a very low consumption of $S_2O_3^{2-}$. Starvation of the trichome bundles for 45 hours in the presence of NO_3^- did not enhance this consumption rate. Accumulation of $S_2O_3^{2-}$ during starvation of disrupted trichome bundles indicates that *Thioploca* cells may not be responsible for the observed accumulation in previous experiments. At present, due to variations in the measurements, it cannot be decided whether or not *Thioploca* produces $S_2O_3^{2-}$ as an intermediate

Sulfate reduction rates measured in sediments from station 7 at the time of sampling were approximately 30 mmol m^{-2} day^{-1} (Strotmann, pers. com.). If all sulfide produced from this reduction was subsequently oxidized by the *Thioploca* mats then *Thioploca* cells should be able to oxidize sulfide with a rate of 20.4 nmol min^{-1} mg $protein^{-1}$ (see Materials and Methods). In comparison, Ferdelman *et al.* (1997) measured an average SO_4^{2-} reduction rate of 17.5 mmol m^{-2} day^{-1}, indicating that *Thioploca* should be able to oxidize sulfide with a rate of 11.8 nmol

min^{-1} mg $protein^{-1}$ (see Materials and Methods). The average sulfide oxidation rate observed during our experiments was 5 nmol min^{-1} mg $protein^{-1}$, which increased to 10.7 nmol min^{-1} mg $protein^{-1}$ after starvation. Compared to the above mentioned reduction rates, this oxidation rate of *Thioploca* could be responsible for 25-91 % of the observed SO_4^{2-} reduction rates measured in the sediments. This indicates that *Thioploca* species may be able to oxidize the majority of the sulfide produced in the sediment of the continental shelf. These data are in agreement with observations by Ferdelman *et al.* (1997), who found an oxidation capacity for *Thioploca* of 35 % of the sulfide production in the sediment.

4.3 C-metabolism

Addition of ^{14}C-labeled bicarbonate resulted in an incorporation rate of 0.4-0.8 nmol min^{-1} mg $protein^{-1}$. The presence of sulfide did not increase the incorporation rate significantly. The measured SO_4^{2-} production rate (generated from internal sulfur) was 2-3 nmol min^{-1} mg $protein^{-1}$, which is equivalent to an average of 1.3 nmol min^{-1} mg dry $weight^{-1}$, assuming that 50% of dry weight is protein. From these data we can predict the CO_2-fixation rate, assuming that 12.5% of the electrons produced go to CO_2-fixation (assuming a yield of 8 g dry weight $(mol\ sulfide)^{-1}$, (Nelson et al., 1986; Timmer-ten Hoor, 1981). The oxidation of sulfur to SO_4^{2-} produces 6 electron equivalents. Given the fact that CO_2 reduction to biomass (dry weight) requires 4 electron equivalents, the predicted rate of CO_2 fixation would be (0.125 x (6/4) x 1.3 =) 0.24 nmol min^{-1} mg dry $weight^{-1}$. This rate is equivalent to 0.49 nmol min^{-1} mg $protein^{-1}$ (assuming that 50% of dry weight is protein), which is the rate observed, suggesting that *Thioploca* species can grow autotrophically using the internally stored sulfur and NO_3^- for energy generation. Results obtained with microautoradiography confirm earlier qualitative experiments by Maier and Gallardo (1984) and indicate that the CO_2 fixation measured can be attributed to *Thioploca* trichome bundles and not to epibiontic bacteria. Ferdelman *et al.* (1997) measured a CO_2 fixation rate in cleaned *Thioploca* suspensions of 2400 ± 700 nmol day^{-1} g wet $weight^{-1}$. Assuming that wet weight of trichomes is 10% of the wet weight of sheaths and trichomes (Schulz et al., 1996), that 10% of the wet weight of trichomes is cytoplasm, that 24 % of the wet weight of the cytoplasm is dry weight and that 50% of dry weight is protein (see calculations in Materials and Methods), then the fixation rate was estimated to be 1.4 ± 0.4 nmol min^{-1} mg $protein^{-1}$. This rate is approximately three times as high as the rate observed in our study.

Experiments performed with ^{14}C-labeled acetate in the absence of sulfide resulted in an uptake rate of approximately 0.4 nmol min^{-1} mg $protein^{-1}$. Microautoradiography showed that epibiontic bacteria also incorporated acetate, but the majority of the label (> 50%) was taken up

by trichomes. Labeling experiments performed with *Thiobacillus neapolitanus* showed that obligate autotrophs are able to incorporate acetate via an incomplete TCA-cycle, lacking the enzyme α-ketoglutarate dehydrogenase (Kuenen and Veldkamp, 1973), resulting in an acetate incorporation rate of 20-30% of the CO_2 fixation rate. However, for the *Thioploca* trichome bundles the acetate uptake rate was approximately equal to the CO_2 fixation rate, which strongly suggests that *Thioploca* species are facultative chemolithoautotrophs, as previously shown for a marine *Beggiatoa* strain (Hagen and Nelson, 1996) and as has also been suggested for the large vacuolated *Beggiatoa* spp. from the Guaymas Basin (Nelson et al., 1989). Production of ^{14}C-labeled CO_2 was not observed after 2-^{14}C-acetate addition, suggesting that acetate, under these conditions, was used only as a C-source for cell carbon, since total oxidation of acetate for energy would release ^{14}C-labeled CO_2. Since *Thioploca* has internally stored sulfur, which is available as energy source, it would be the most beneficial, strategically, to use acetate as the primary C-source. This economic use of energy and carbon sources is typical for mixotrophic growth (Gottschal and Kuenen, 1980).

Labeling experiments with bicarbonate and acetate followed by microautoradiography showed localization of the label along the transverse walls, indicating the presence of the central vacuole.

The ecophysiological experiments presented here indicate that *Thioploca* is a facultative chemolithoautothroph, capable of fixing CO_2 and of assimilating available acetate when sulfur or sulfide is present as an energy source. This use of acetate as a carbon source when other substrates are present as an energy source, is typical behavior for organisms capable of mixotrophic growth. In spite of its ability to rapidly respond to fluctuations in both NO_3^- and sulfide, its metabolic strategy seems to be geared to continuous, but extremely slow growth, which is apparently unaffected by such fluctuations. Indeed, the large reservoir of both NO_3^- (average 160 mM) and S^0 (200 nmol mm^{-3}) would indicate a turnover time of NO_3^- and S^0 of 8-10 days. Based on the observed rate of autotrophic CO_2-fixation, *Thioploca* would grow with a doubling time of 69-139 days under the laboratory conditions tested (0.4-0.8 nmol CO_2 min^{-1} mg $protein^{-1}$ is equal to 0.4-0.8 nmol C min^{-1} mg C dry $weight^{-1}$, assuming that 50% of dry weight is carbon. 1 mg C is 0.08 mmol C and, thus, it would take 69 to 139 days to incorporate this amount). Assuming that *Thioploca* can grow mixotrophically on acetate, this doubling time could be increased to 26-52 days. Although this may be an underestimate, such a rate coincides with the observed increase in biomass of 1 g wet weight m^{-2} day^{-1} as has been observed for Station 6 by H. N. Schulz (pers. com.). This increase would lead to a doubling time of approximately 70 days, assuming an average of 85 g wet weight m^{-2} for trichomes without

sheaths (see Materials and Methods). In general, however, we should remember that samples used in this study were mixed populations and therefore, differences in activities between the two species used, may occur.

In spite of its low growth rate, the evidence presented here shows that *Thioploca* is one of the major players in sulfur and nitrogen cycling of the sediment along the West Coast of South America.

Acknowledgments

This research was part of a joint project between the Max Planck Institute of Marine Microbiology and the University of Concepción, Chile. We greatly appreciate all the enthusiasm, help and support that we received from the people at the Estación de Biologia Marina in Dichato, the crew of the *Kay Kay*, the staff from the University of Concepción who helped us with the ^{14}C-scintillation counter and all the members of the scientific party present. The study was supported by the Max Planck Society, the University of Concepción, Delft University of Technology, the Netherlands Organization for Scientific Research (NWO project R83-151), the Woods Hole Oceanographic Institution (WHOI Contribution No. 9730), the FONDAP-HUMBOLDT Program and the National Science Foundation (OCE 94-15985). Furthermore, we would like to thank the reviewers for their many helpful suggestions to improve the manuscript.

Sulfide Oxidation and Speciation of Sulfur Intermediates in Marine Environments

DISSERTATION

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CHAPTER 4

Ecology of *Thioploca* spp.: NO_3^- and S^0 Storage in Relation to Chemical Microgradients and Influence on the Sedimentary Nitrogen Cycle

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Abstract

Microsensors, including a recently developed NO_3^--biosensor, were applied to measure O_2 and NO_3^- profiles in marine sediments from the upwelling area off Central Chile and to investigate the influence of *Thioploca* spp. on the sedimentary nitrogen metabolism. The studies were performed in undisturbed sediment cores incubated in a small laboratory flume to simulate the environmental conditions of low O_2, high NO_3^-, and bottom water current. Upon addition of NO_3^- and NO_2^-, *Thioploca* spp. exhibited positive chemotaxis and stretched out of the sediment into the flume water. In a core densely populated with *Thioploca* the penetration depth of NO_3^- was only 0.5 mm, and a sharp maximum of NO_3^- uptake was observed 0.5 mm *above* the sediment surface. In *Thioploca* spp. poor sediment, NO_3^- was detectable down to 2 mm depth, and the maximum consumption rates were observed, *within* the sediment. No chemotaxis towards N_2O was observed which is consistent with the observation that *Thioploca* does not denitrify, but reduces intracellular nitrate to ammonium. Measurements of the intracellular $<NO_3^->$ and elemental sulfur ($<S^0>$) pools in *Thioploca* filaments from different depths in the sediment revealed a weak, negative correlation between the two storage compounds. Although the $<NO_3^->/<S^0>$-ratio was highly variable among individual filaments, the average concentration calculated from all filaments present in a given depth interval was remarkably constant down to about 10 cm depth. This apparent contradiction may be explained by a rapid vertical shuttling of *Thioploca* with the consequence that in a given depth as well downward-moving $<NO_3^->$-rich, $<S^0>$-poor as upward moving $<NO_3^->$-poor, but $<S^0>$-rich filaments were present. Intracellular $<NO_3^->$ was found down to a depth of 16 cm, providing additional support for the concepts of vertically shuttling *Thioploca* and the NO_3^- transport into the sediment.

1. Introduction

Although the capability of microorganisms to oxidize reduced sulfur compounds with nitrate as the electron acceptor has been known for a hundred years (Beijerinck, 1904) and several pure cultures have been obtained and studied (e.g. Taylor et al., 1971; Timmer ten Hoor, 1975), only little is known about the ecological significance of this type of metabolism. For instance, the first report showing a clear coupling between the sulfur and the nitrogen cycle in the environment was sulfide driven denitrification at the oxic-anoxic interface in the water column of the central Baltic Sea (Brettar and Rheinheimer, 1991; 1992).

It was recently discovered that sulfide-oxidizing bacteria of the genus *Thioploca* possess large nitrate filled vacuoles (Fossing et al., 1995). *Thioploca* is highly abundant in the shelf

sediments along Peru and Chile (Froelich et al., 1988; Gallardo, 1977; Henrichs and Farrington, 1984; Rosenberg et al., 1983) and it was suspected that these organisms play a major role in coupling the biogeochemical cycles of nitrogen and sulfur in upwelling areas (Fossing et al., 1995). The observation of benthic *Thioploca* filaments in the upwelling area of the Arabian Sea (Levin et al., 1997) and the finding of both *Thioploca* and the spherical, nitrate storing bacterium *Thiomargarita* off Namibia (Schulz et al., 1999) support this conclusion. Nitrate-storing sulfide-oxidizing bacteria have also been observed at hydrothermal springs, vents (Mattison et al., 1998; McHatton et al., 1996), and cold seeps (McHatton et al., 1996), in organic rich sediments of lakes (Nishino et al., 1998) and a brackish fjord in Denmark (L. P. Nielsen, unpubl. data).

Currently, none of the nitrate storing sulfur bacteria is in pure culture and alternative methods have to be applied to study their physiology and ecology. Enzyme preparations from *Beggiatoa* samples showed high nitrate reductase and ribulose-1,5-bisphosphate carboxylase-oxygenase activity and provide the first biochemical evidence for the use of nitrate as electron acceptor for sulfide oxidation and chemoautotrophic growth (McHatton et al., 1996). Incubation experiments with partially purified *Thioploca* filaments revealed that sulfide (ΣH_2S) is first rapidly oxidized to intracellular, elemental sulfur ($<S^0>$) which is then further oxidized to sulfate (SO_4^{2-}) in a second, independent step. Intravacuolar nitrate ($<NO_3^->$) serves as the electron acceptor and was reduced to ammonium (NH_4^+) (Otte et al., 1999). Radiolabeled bicarbonate ($H^{14}CO_3^-$) and (2-^{14}C)acetate are assimilated indicating that *Thioploca* is a facultative chemolithoautotroph capable of mixotrophic growth (Maier and Gallardo, 1984; Otte et al., 1999). Whole core incubations in small laboratory flumes have helped to unveil the chemotactic behavior of *Thioploca* under changing environmental conditions. *Thioploca* showed positive chemotaxis towards nitrate and low sulfide concentrations (<100 µM) but a phobic reaction towards oxygen and high sulfide concentrations (Huettel et al., 1996). These observations together with mostly vertical orientation of *Thioploca* sheaths in the sediment (Schulz et al., 1996), led to the suggestion that *Thioploca* shuttles up and down between NO_3^-rich bottom water and H_2S containing sediment layers (Fossing et al., 1995; Huettel et al., 1996).

The samples for this study were collected at stations within and off the Bay of Concepción in central Chile where the species composition and annual dynamics of the *Thioploca* population is known from a previous study (Schulz et al., 2000). On the shelf the community was mainly composed of *T. araucae*, *T. chileae* and a yet undescribed form of *Thioploca* with much shorter cells (Schulz et al., 2000). This so-called short-cell morphotype (SCM) is usually found in deeper sediment layers than the two other species and is characterized by rounded cells and a cell length/diameter-ratio of ≤0.48. The SCM is closely related but not identical to the known *Thioploca* species as revealed by partial 16S rDNA analysis (Schulz, 1999). Within the Bay of Concepción large vacuolated filaments cover the sediment surface

during part of the year (Schulz et al., 2000). They are, besides the lack of sheaths, phenotypically and phylogenetically almost identical to *T. araucae* (Schulz et al., 2000; Teske et al., 1999).

The aim of this study was to gain information on the ecology of *Thioploca* and its influence on the nitrogen and sulfur cycles in the habitat. By the use of microsensors we show that the nitrate uptake of the sediment is strongly influenced by *Thioploca*. Chemotaxis towards inorganic nitrogen compounds was studied and measurements of intracellular $<S^0>$ and $<NO_3^->$ concentrations were used to test the concept of a vertical shuttling between the nitrate rich bottom water and sulfidic deep sediment layers. We also demonstrate that *Thioploca* transports NO_3^- down to a sediment depth of at least 16 cm.

2. Methods

2.1 Study area

The continental shelf region off the Concepción Bay (central Chile) is characterized by intense seasonal upwelling. Between austral late spring and early fall southern and southwestern winds prevail and the northward flowing Subantarctic Surface water is forced off the coast leading to upwelling of Equatorial Subsurface water (ESSW) from the Poleward Undercurrent at 100 to 400 m (Strub et al., 1998). The ESSW is characterized by high salinity (34.4 - 34.8 ‰), low temperature (8.5 - 10.5 °C), low oxygen (< 20 µM), but high nitrate (about 25 µM) and nutrient concentrations (Strub et al., 1998). Upwelling off central Chile is intermittent and usually lasts between 2 and 7 days (Hill et al., 1998). Primary and secondary productivity greatly increases when the nutrient rich water is transported up into the euphotic zone. For the coastal upwelling area off central Chile a primary production of 9.6 g C m^{-2} d^{-1} has been reported (Fossing et al., 1995), which is one of the highest observed in marine environments. Due to the lack of oxygen and sufficient amounts of alternative electron acceptors, sedimentary organic matter is almost exclusively degraded by sulfate-reducing bacteria (Thamdrup and Canfield, 1996). The sulfate reduction rates reported for this area are among the highest observed in coastal margins, but free sulfide concentrations in these sediments are surprisingly low, indicating an efficient reoxidation of sulfide (Ferdelman et al., 1997; this thesis Chapter 5).

2.2 Sampling and site description

During January - February 1997 we repeatedly sampled sediment from three stations within and off the Bay of Concepción (see Chapter 3, Fig. 1). The sediment was collected from the research vessel *Kay Kay* of the University of Concepción (Concepción, Chile) by means of a small gravity corer. The cores were stored onboard at 4 °C in a refrigerator and were transported to the Marine Biological Station of the Univ. Concepción in Dichato.

Station 4 (36° 38' 08" S; 073° 02' 03" W) was 24 m deep and located within the bay (see Chapter 3, Fig. 1). The sediment at this station was highly sulfidic (up to 1200 µM; see Chapter 5) and was uniformly black below the brownish uppermost 3-4 mm. The top 4.5-5 cm of the sediment was flocculent ooze with mass accumulation of sheathless, filamentous sulfur bacteria. The sediment of Station 7 (36° 36' 05" S; 073° 00' 06" W; 32 m deep) at the mouth of the bay, was covered by a brown, spongy layer of 1.5 cm which was densely populated by *Thioploca* spp.. Below this layer was a 0.4 cm thick band of black iron sulfide followed by gray-brownish sediment underneath. Burrows of sediment dwelling animals were observed. The sediment of Station 18 (35° 30' 08" S; 073° 07' 06" W; 88 m deep) had similar structure with a spongy layer of 0.5 - 1 cm, a thin black layer of 0.2 - 0.3 cm, and then gray sediment below. *Thioploca*, however, was much less abundant, and no animal burrows were observed. The concentration of free sulfide was at both stations <6 µM down to a depth of 20 cm. During the sampling period the bottom-water concentration of O_2 was <2 to 7 µM at all stations. The NO_3^- concentrations were about 6 µM at St. 4, and 7-24 µM at St. 7 and 18 (Schulz et al., 2000).

2.3 Microsensor measurements and flux calculations

A Clark-type O_2 microsensor with a guard cathode was used to measure oxygen microprofiles (Revsbech, 1989). Nitrate was measured with a micro-biosensor consisting of an electrochemical N_2O microsensor surrounded by an outer casing (Larsen et al., 1997). A 100-200 µm long reaction chamber was formed between the tip of the internal N_2O sensor and the ion-permeable membrane at the tip of the outer casing. A N_2O reductase-deficient culture of *Agrobacterium* immobilized in the reaction chamber transformed NO_3^- and NO_2^- to N_2O, which was detected by the N_2O microsensor. Due to this design the microsensor measured the combined NO_3^-, NO_2^- and N_2O concentrations in a sample. The N_2O microsensor was constructed like a normal O_2 microsensor (Revsbech, 1989), but the cathode was plated with silver and the electrolyte consisted of 0.5 M NaOH and 0.5 M KCl. The N_2O microsensor was polarized at -1.2 V against an Ag/AgCl anode immersed in the electrolyte. The current from the microsensor was measured with a custom-made pA-meter and recorded on a strip chart recorder. The active silver surface of the N_2O sensing cathode is also sensitive towards H_2S. However, the concentrations of free sulfide in the top centimeters of St. 7 and 18 sediment was usually <1 µM, and hence no interference with H_2S was anticipated. Calibrations and measurements were done at the same temperature. The detection limit of the sensor was about 3 µM NO_3^-, and the linear range was between 3-70 µM NO_3^-. The sensitivity for N_2O was about 3 times better than for NO_3^-, the signal was linear up to N_2O at least 1000 µM. The response time for 90% signal intensity was about 50 s and a resting time of one minute was used for each depth step in a profile. The position of the sediment surface was determined for each profile using a dissection microscope (10-50x magnification).

Fluxes across the sediment-water interface were calculated from the upper linear part of the microsensor profiles by Fick´s first law of diffusion. J = -D * dC / dx, where J is the flux in μmol cm^{-2} s^{-1}, D is the diffusion coefficient (cm^2 s^{-1}) and dC / dx is the concentration gradient (μmol cm^{-3} cm^{-1}). Tabulated diffusion coefficients were recalculated for the *in situ* temperature and salinity (Broecker and Peng, 1974; Li and Gregory, 1974). Since the sediment was very porous in the top centimeter, the same diffusion coefficients of 1.8 and $1.41*10^{-5}$ cm^2 s^{-1} for O_2 and NO_3^- respectively, were used.

2.4 Experimental set-up

In order to simulate the environmental conditions prevailing off the coast, measurements were done in a small flow chamber where deaerated surface water from Station 7 was circulated. A small aquarium pump was used to create flows of approximately 4-6 cm s^{-1} about 2 cm above the sediment surface. The flow chamber consisted of two horizontal Plexiglas plates that were separated with a spacer. Both Plexiglas plates contained a central hole (50 cm^2). A sediment core was brought into the flow through the bottom hole and the microsensors were introduced through the hole in the upper plate. A dissolved O_2 concentration of about 5 μM was maintained in the circulating water by adjusting the area of air-exposed seawater at the top hole. The water in the set-up was kept at the *in situ* temperature of 12 °C by a thermostated circulating cooler.

2.5 Extraction and analysis <NO_3^-> and <S^0> in Thioploca

Thioploca filaments from different sediment depths of St. 7 were individually picked and aligned in a film of seawater on a microscope slide. Length (l) and diameter (d) of the filaments were determined, and the biovolume (V) was calculated ($V = \pi l d^2 / 4$). According to the cell length to diameter ratio they were identified as *T. araucae*, *T. chileae* or as "Short cell morphotype" (Schulz et al., 2000). The filaments were then briefly washed in seawater and dried on a glass capillary to break the cells. Nitrate was extracted by dipping the dried filaments in demineralized water and analyzed by a miniaturized version of the cadmium reduction method (Grasshoff et al., 1983). From the same filament, zerovalent sulfur was extracted with 50 μl methanol. The complete dissolution of sulfur globules was verified by extraction time-series and light microscopy. Elemental sulfur in the extract was quantified as cyclo-octasulfur (S_8) by high performance liquid chromatography (HPLC). Separation was done on a Zorbax ODS-column (125x4 mm, 5 μm; Knauer, Germany) with methanol (100%; HPLC grade) as eluent at a flow rate of 1 ml min^{-1}. S_8 eluted after 3.5 minutes and was detected at 265 nm. The detection limit was about 1 μM and the analytical precision of the method was 0.5 % RSD. The elemental sulfur in the bulk sediment was extracted from Zn-preserved samples with pure methanol for 16 h on a rotary shaker; the sediment to extractant ratio was about 1:20 (ww/vol). Quantification of S^0 in the filtered (0.45 μm) extracts was done with the same setting as described above.

Statistical treatment. The correlation between $<NO_3^->$ and $<S^0>$ was determined and tested for significance by the method of Spearman (Sachs, 1997). The $<NO_3^->/<S^0>$-ratios of filaments from different depth intervals were tested for significant differences by using the H-test of Kruskal and Wallis (Sachs, 1997).

3. Results and Discussion

3.1 Nitrate microprofiles in seawater

Nitrate has been measured in biofilms and lake sediments by liquid ion exchanger (LIX) based microsensors (de Beer and Sweerts, 1989; Schramm et al., 1999; Sweerts and de Beer, 1989). Similar measurements in marine environments were previously not possible due to the interference of Cl^- ions. A recently developed NO_3^- biosensor was used in this study to measure microscale profiles of NO_3^- in sediments from the upwelling system off Central Chile. Measurements were conducted in a small laboratory flume where freshly collected sediment was incubated under *in situ* conditions. To illustrate the spatial and temporal variability, several profiles from St. 18 are shown in Fig. 2.

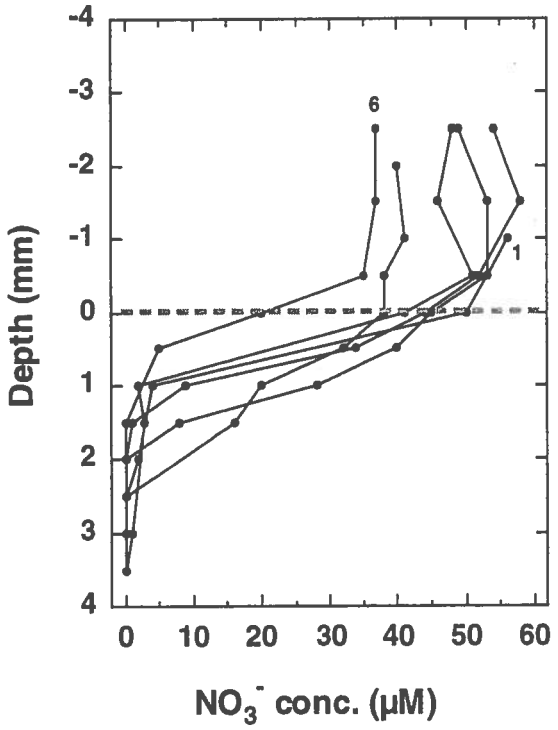

Figure 2: Spatial and temporal variability of NO_3^- microprofiles in sediment inhabited by only few *Thioploca* filaments (St. 18).

The penetration depth of nitrate into this highly active sediment was only 1 to 2.5 mm, which is in the same range as found in organic-rich lake sediments (Sweerts and de Beer, 1989). The nitrate concentration in the flume water decreased between 1 and 6 due to the activity of denitrifying bacteria. However, because of biofilm formation on the flume and tube walls, calculations of NO_3^- uptake of the sediment based on the NO_3^- decrease over time are, unfortunately, not possible. However, diffusional uptake calculations can be performed from the nitrate gradient at the sediment water interface.

3.2 Influence of Thioploca on profiles and uptake rates

Huettel et al. (1996) suggested that filament protrusion may be a strategy to overcome the diffusion limitation to nitrate uptake imposed by the boundary layer, and that *Thioploca* may thereby outcompete nitrate consuming bacteria in the sediment. To test this hypothesis we incubated sediment from two different stations in the flume under similar conditions as above and measured oxygen and nitrate profiles.

At the time of sampling, the sediment of St. 7 was densely populated by *Thioploca* with a total biomass of 44 g ww m^{-2} (Schulz et al. 2000) an the filaments stretched out of the sediment when nitrate was present in the flume water. *Thioploca* was much less abundant in the

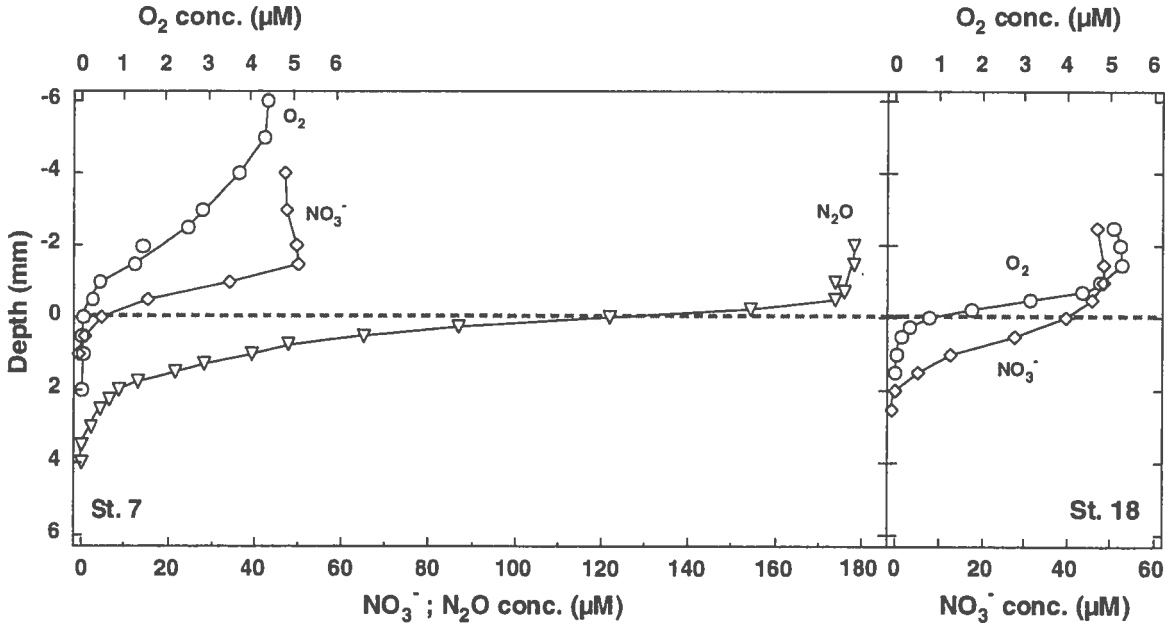

Figure 3: Laboratory flume measurements of O_2, NO_3^- and nitrous oxide (N_2O) in sediment with a high (St. 7) and very low density of *Thioploca* (St. 18). Nitrous oxide was not measured at St. 18. A broken line indicates the sediment-water interface.

second core from St. 18 where the wet biomass was only 9 g m^{-2} (Schulz et al., 2000). Protruding filaments were only sporadically observed in the core studied. This difference in the *Thioploca* spp. abundance was clearly reflected in the O_2 and NO_3^- profiles (Fig. 3). The profiles of St. 7, measured in the vicinity of Thioploca filaments, were oddly shaped and O_2 and NO_3^- hardly reached to the sediment surface. Since the filaments protruded into the flume water, maximum NO_3^- uptake rates occurred *above* the sediment surface (Fig. 4) and NO_3^- penetration was only 0.5 mm. Station 18 showed an O_2 microprofile normal for marine sediments, with a diffusive boundary layer thickness of about 1 mm and a maximal O_2 penetration depth of 1 mm. Nitrate penetrated about 2 mm into the sediment and the linear range of the gradient (Fig. 3) and maximal uptake rates were both *within* the sediment (Fig. 4).

Interestingly, the diffusive boundary layer at St. 7 was >1.5 mm, considerably thicker than at St. 18 (Fig. 3). A likely explanation for this could be that *Thioploca* filaments protruding from the sediment impede the water flow, which leads to a thickening of the boundary layer and thus to a lower diffusive exchange across the sediment-water interface. Thus, the oxygen uptake at St. 7 only 0.26±0.11 mmol m^{-2} d^{-1} (n = 6) compared to 0.74±0.13 mmol m^{-2} d^{-1} (n = 6) at St. 18. The calculated diffusive NO_3^- uptake at St. 7 was 5.45±1.17 mmol m^{-2} d^{-1} (n = 4), about 55% higher than at St. 18 (3.53±0.76 mmol m^{-2} d^{-1}; n = 5). This is consistent with the observations by Huettel et al. (1996) of a 10-fold increase, from 2 to 20 mmol m^{-2} d^{-1} of total NO_3^- uptake, when the *Thioploca* filaments stretched out of the sediment. The total NO_3^- uptake of sediment a bacteria is thus significantly higher than calculated on the basis of 1-dimensional microgradients. In summary, *Thioploca* spp. have a major influence on the sedimentary NO_3^- metabolism. They penetrate up through the diffusive boundary layer, cause a reduced NO_3^- penetration depth, move the maximum NO_3^- uptake upwards, and increased the areal NO_3^- uptake rate.

An interesting effect of the *Thioploca* community might be that nitrate-reducing bacteria in the sediment are outcompeted for NO_3^-, and that denitrification consequently plays a minor role in sediments densely inhabited by these filamentous sulfur bacteria. The situation, however, is probably more complex as indicated by denitrification measurements. Despite the presence of *Thioploca* spp. denitrification rates of 4.5 and 9 mmol m^{-2} d^{-1} were determined for St. 7 by adding 100 µM $^{15}NO_3^-$ to the flume water (L. P. Nielsen). These values are somewhat higher than typically observed in normal coastal sediments (Herbert, 1999 and refs. therein) and suggest only incomplete suppression of denitrification. Nitrate for denitrification may be supplied by leakage from *Thioploca* filaments or via advective transport of bottom water into the sediment. Advective transport becomes progressively more important with increasing flow velocities (Forster et al., 1996) and may indeed have been an important process because the top 1-2 cm of the sediment had a spongy consistency and was very porous due to the *Thioploca*-

mat. (Reimers, 1982). Furthermore, the in situ velocity of the sediment-near bottom water may well exceed the 5 cm s^{-1} that we have used in our study.

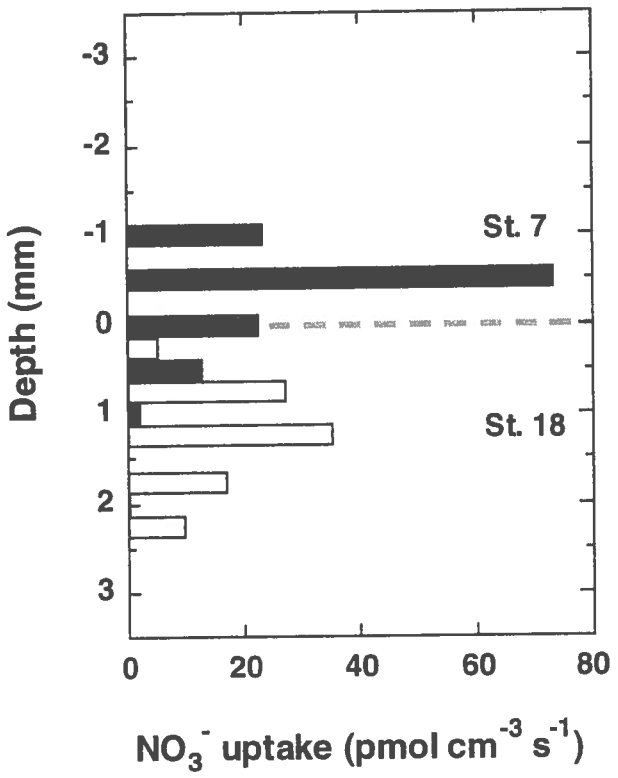

Figure 4: Vertical distribution of NO_3^- uptake rates in sediment with a high (St. 7) and very low density (St. 18) of *Thioploca* filaments. The broken line indicates the sediment water interface.

3.3 Response of Thioploca filaments towards NO_2^- and N_2O

The chemotactic behavior of *Thioploca* spp. towards O_2, NO_3^- and ΣH_2S was studied by Huettel and coworkers (1996). However, also NO_2^- as N_2O are intermediates and byproducts of nitrification and denitrification processes (Herbert, 1999; Knowles, 1982) and can be found in the oxygen minimum zone (OMZ) of upwelling areas (Codispoti et al., 1986; Copin-Montégut and Raimbault, 1994). We studied whether *Thioploca* spp. show chemotaxis towards NO_2^- and N_2O and whether they can utilize them as electron acceptors using cores from St. 7 and adding NO_2^- or N_2O to nitrate-free flume water. Upon addition of 10 µM NO_2^- the number of protruding filaments and the total length of filaments rapidly increased and reached a maximum after 1.2 hours, when the NO_2^- concentration in the flume was 2.6 µM (Fig. 5). Below that concentration the filaments began to retreat until a steady state was achieved similar to that before NO_2^- addition.

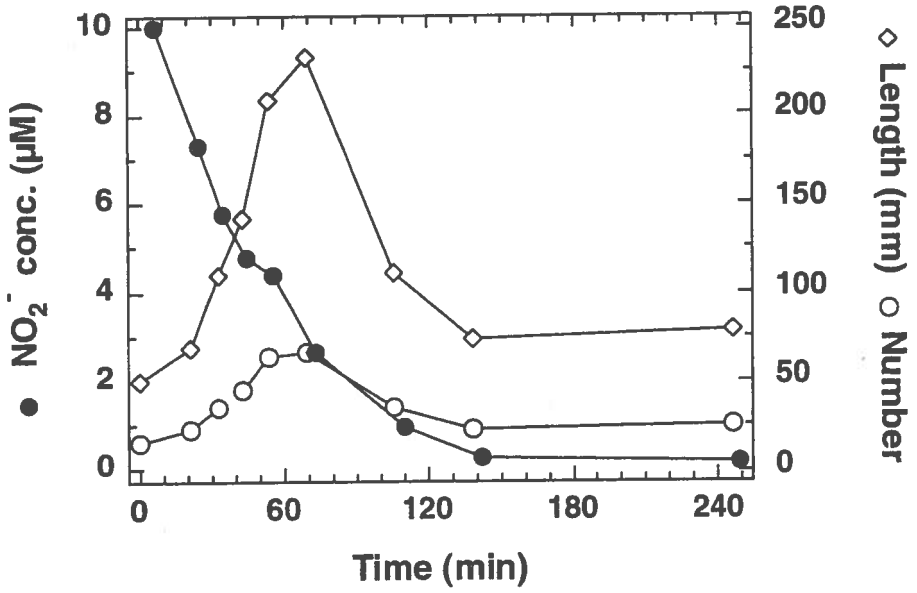

Figure 5: Response of *Thioploca* towards addition of 10 µM NO_2^- to the flume water as indicated by the *number* of filaments protruding >2 mm out of the sediment and by the total *length* of all filaments exposed to flume water.

In contrast to NO_3^- and NO_2^-, *Thioploca* filaments did not show chemotaxis towards N_2O suggesting that nitrous oxide may not be used as an electron acceptor for sulfide oxidation. Nitrous oxide is the obligate precursor for dinitrogen formation during denitrification and can be used by most, though not all, denitrifying bacteria as electron acceptor (Zumft and Kroneck, 1990). Reduction of N_2O to ammonium has never been reported. The absence of a response towards N_2O is therefore consistent with the finding of Otte and coworker (1999) that ammonium rather than dinitrogen, as previously assumed (Fossing et al., 1995), is the terminal product of nitrate reduction.

Microprofiles of N_2O were measured during the chemotaxis experiment, and an average profile (n = 3) is included in Fig. 3. Since Thioploca did not stretch out from the sediment the N_2O profile exhibited a regular diffusive boundary layer of about 0.5 mm. However, even without the contribution of *Thioploca* spp., N_2O is consumed rapidly (15.1±1.5 mmol m^{-2} d^{-1}) within the first 3.5 mm of the sediment, demonstrating the potential for sedimentary dinitrogen formation.

3.4 Internal <S^0> and <NO_3^-> concentrations

A concentration range of 150 to 500 nmol mm^{-3} (= mM) has been reported for the intracellular nitrate (<NO_3^->) content of *Thioploca* cells (Fossing et al., 1995), but to date nothing is known about the variability within the different species nor about the <NO_3^->/<S^0>-ratio within individual filaments and whether it changes with depth. Additionally, *Thioploca*

filaments have been found down to 26 cm depth (Schulz et al., 2000) but it was not clear, whether they were still alive and contained $<NO_3^->$. Therefore, *Thioploca* filaments were collected from different depths of a core from St. 7, and the internal concentrations of $<NO_3^->$ and $<S^0>$ were determined. The statistical analysis of the results from the depth intervals 0-1 cm, 1-2 cm, 2-3 cm, 3-4 cm, 4-7 cm, and 7-10 cm did not indicate significant differences in the $<NO_3^->/<S^0>$-ratios between the different sections even for $\alpha = 0.1$. With the same statistical method it was shown that the two lowest intervals from 10-13 cm and 13-16 cm, although not different from each other, were different from the first 6 intervals with a high probability ($\alpha = 0.001$). Based on these results, the measurements of internal $<NO_3^->$ and $<S^0>$ from 0 to 10 cm and from 10 to 16 cm depth, respectively, are grouped (Fig. 6A, B).

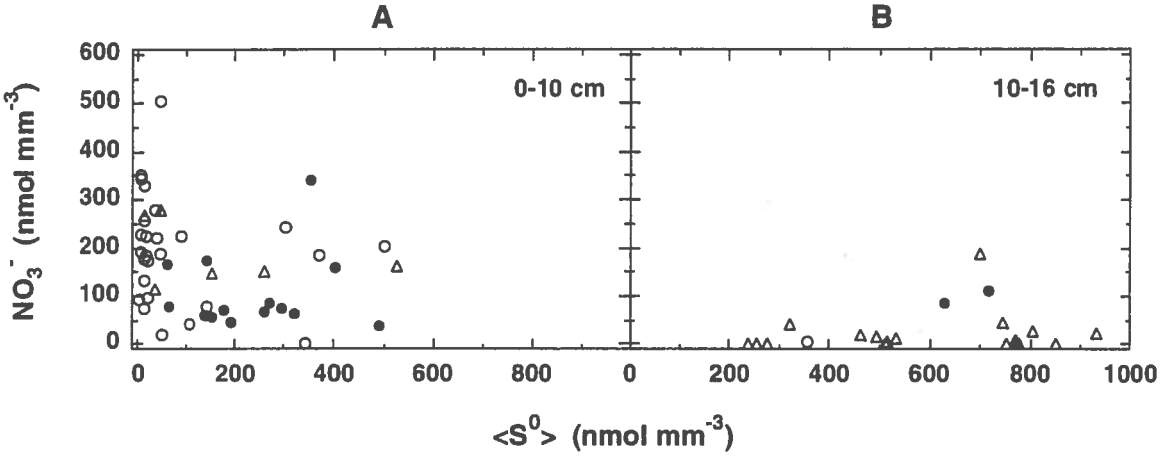

Figure 6: Concentrations of $<NO_3^->$ and $<S^0>$ in *Thioploca* collected from different depths of sediment from Station 7. Open circles: *T. araucae*; closed circles: *T. chileae*; open triangles: short cell morphotype (SCM).

In the upper part (0-10 cm) of the core *T. araucae* (57%) and *T. chileae* (30%) were the dominant mat forming species (Fig. 6A), with the rest being short-cell morphotype thioplocas (SCM). The SCM were more abundant (81%) in the deeper section, which is consistent with observations of Schulz et al. (2000) who found maximum biomass of *T. araucae* and *T. chileae* close to the sediment water interface, whereas the SCM was most abundant below 7 cm. The difference in the species composition was also reflected in the $<NO_3^->$ and $<S^0>$ concentrations. Whereas $<NO_3^->$ and $<S^0>$ varied over similar concentration ranges (up to ≈500 nmol mm^{-3}) in the upper section (Fig. 6A), the values were significantly shifted towards lower $<NO_3^->$ and higher $<S^0>$ concentrations in the 10-16 cm section. Both the SCM and *T. chileae* stored $<S^0>$ up to ≈800 nmol mm^{-3}. The maximum $<S^0>$ concentration determined for *T. araucae* was only 355 nmol mm^{-3} (Fig 6B), which nicely corresponded to the lower volume ratio between cytoplasma and vacuole. Whereas SCM and *T. chileae* have both a C/V-ratio of

0.47, it is only 0.23 in *T. araucae*. Despite the high variability of the $<NO_3^->$ and $<S^0>$ concentrations, a weak, but statistically significant negative correlation was observed ($r_{Spearman}$ = -0.31; n = 50, α = 0.05) between $<NO_3^->$ and $<S^0>$.

Based on the results from chemotaxis experiments, it was concluded that *Thioploca* filaments are shuttling between the nitrate rich bottom water and sulfide-containing deeper sediment layers (Fossing et al., 1995; Huettel et al., 1996). Such behavior would imply that filaments with a high $<NO_3^->/<S^0>$-ratio are predominantly found at the sediment surface, where the vacuole is refilled with NO_3^- and that filaments rich in $<S^0>$ but depleted of $<NO_3^->$ are found in deeper sediment layers. This could only be shown for the SCM thioplocas, where a decreasing $<NO_3^->/<S^0>$-ratio correlated with the sediment depth (r^2 = 0.71). By *T. araucae* and *T. chileae* no significant correlation (α = 0.05) between the $<NO_3^->/<S^0>$-ratio in individual filaments and the sediment depth was observed. The reason for the lack of correlation becomes obvious, when the mean $<NO_3^->$ and $<S^0>$ concentrations of all individuals of a species in a given depth interval are calculated (Fig. 7). Despite the high variability in the individual $<NO_3^->/<S^0>$-ratios the mean concentrations were rather constant down to several centimeters depth. This may be due to a continuous and rapid vertical migration of *Thioploca* filaments in the sediment column, relative to the turnover times of the internal storage products. Consequently, in a given depth both, downward-moving $<NO_3^->$-rich, $<S^0>$-poor filaments and upward-moving $<NO_3^->$ poor, $<S^0>$ rich filaments are present, leading to the observed leveled out concentration profiles (Fig. 7). However, possibly the strongest evidence for a vertical shuttling of *Thioploca* spp. and NO_3^- transport into the sediment comes from the observation that $<NO_3^->$ was also found in significant concentrations (23±44 nmol mm^{-3}; n = 17) in the filaments from 16 cm depth. Calculations based on the turnover time of $<NO_3^->$ (8-10 d; Otte et al., 1999) and a migration velocity of 5 mm h^{-1} (Huettel et al., 1996) showed that the internal reservoir of electron acceptor in *Thioploca* cells is plenty to reach such depths.

Based on the results presented in Fig. 7, one may conclude that at least for *T. araucae* and the SCM it is easier to obtain sufficient NO_3^- than ΣH_2S. Nitrate is taken up by the filaments above the sediment in a stirred medium, where NO_3^- is continuously supplied in high concentrations (about 20-30 µM). In contrast, the ΣH_2S concentration in the sediment of St. 7 was about 1 µM (Chapter 5), and the transport to the filaments is only by diffusion. The supply of ΣH_2S to a single filament may additionally be restricted by the fact that several filaments competing for ΣH_2S inhabit a common diffusion-limiting sheath. Although this must be also true for *T. chileae*, there the average $<S^0>$ content was typically higher than the $<NO_3^->$ content (Fig. 8). This could indicate that the NO_3^- uptake in this species is less efficient or that *T. chileae* is adapted to low ΣH_2S concentrations and hence more competitive for ΣH_2S than *T.*

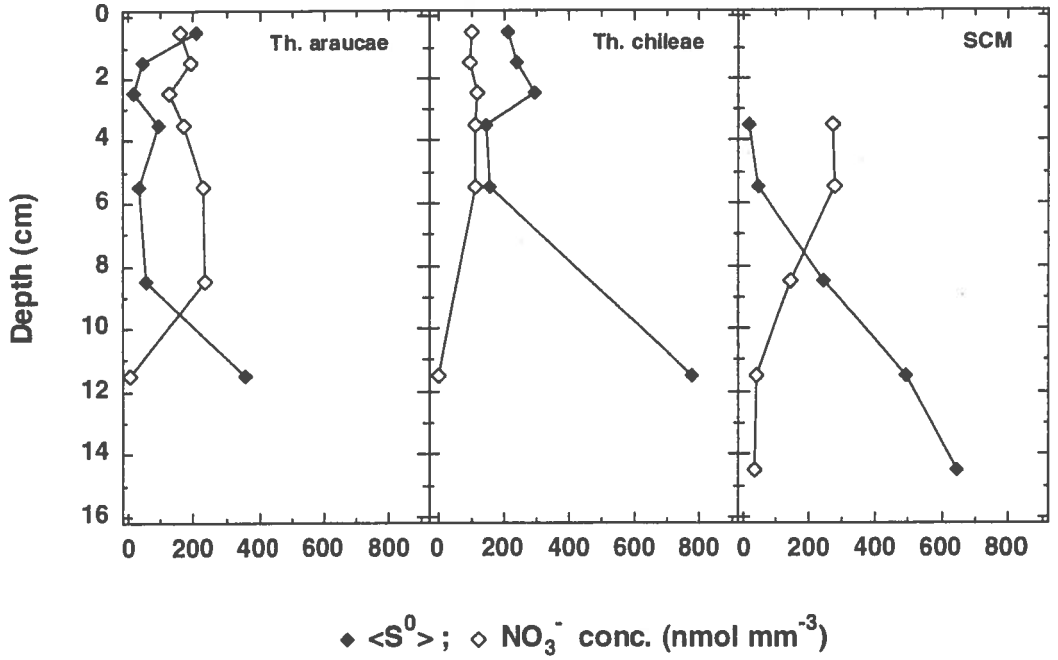

Fig. 7: Vertical distribution of the mean $<NO_3^->$ and $<S^0>$ concentrations in the different *Thioploca* species from St. 7.

araucae and the SCM. The second possibility, however, would be consistent with the higher abundance of *T. chileae* at stations with lower sulfate reduction rates (Schulz et al., 2000).

For comparison, internal $<NO_3^->$ and $<S^0>$ concentrations were also measured in the free-living filaments inhabiting the sediment surface at St. 4 (Fig. 8). Their $<NO_3^->$ content was 42±27 nmol mm^{-3}, i.e. much lower and less variable than in the 0-10 cm section of St. 7 (Fig. 6). It was also considerably lower than reported for *Beggiatoa* spp. from the Monterey Canyon and Guaymas Basin (McHatton et al., 1996). In general, the $<NO_3^->$ and $<S^0>$ concentrations were similar to those of *Thioploca* spp. found below 10 cm at St. 7. This was likely due to the high ΣH_2S content (up to 1200 µM) in this sediment. Based on the finding of a phobic response towards sulfide concentrations >500 µM (Huettel et al., 1996) and the high morphological and phylogenetic similarity to *T. araucae* it was even suggested that these filaments might actually be thioplocas who moved out of their sheaths or do not produce them under the prevailing environmental conditions (Teske et al., 1999).

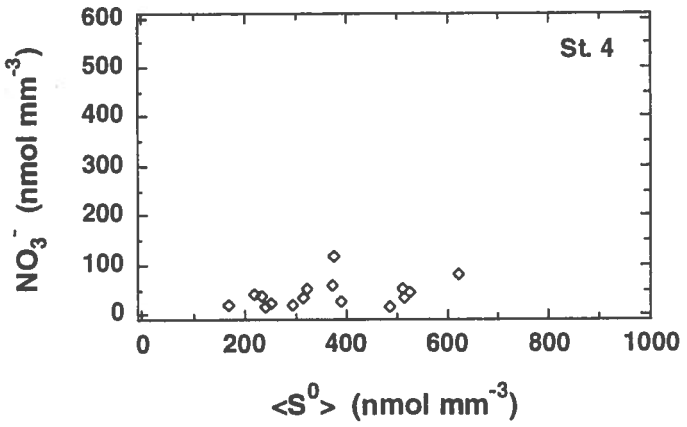

Fig. 8: Concentrations of <NO_3^-> and <S^0> in *Beggiatoa* spp. filaments from the surface of highly sulfidic sediment at St. 4

3.5 Intracellular vs. extracellular pools of S^0 and NO_3^-

In sediments of a Danish fjord and in the Santa Barbara basin it was observed that elemental sulfur was primarily associated with *Beggiatoa* filaments (Schimmelmann and Kastner, 1993; Troelsen and Jørgensen, 1982). In order to quantify the contribution of <S^0> to the total pool of S^0 in the sediment, we manually collected all *Thioploca* filaments present in a core from St. 7 and determined <S^0> and S^0 in the remaining bulk sediment (Fig. 9). In contrast to the two other studies we found that <S^0> made up at most 27% of the total S^0 pool. Furthermore, the two sulfur pools exhibited a different distribution pattern. Whereas <S^0> corresponded to the distribution of *Thioploca* spp. (Schulz et al., 1996) and decreased gradually with depth, the concentration of extracellular S^0 was maximal at a depth of 5 cm. Because the turnover times of the two sulfur pools may be different one can not draw quantitative conclusions about the relative significance of *Thioploca*-associated and chemical sulfide oxidation. However, it demonstrates that other processes contribute to sulfide oxidation and sedimentary S^0 formation. The distribution of chemical species (ΣH_2S, Fe^{2+}, $S_2O_3^{2-}$) in the porewater suggests that reducible iron oxides may be an important oxidant for porewater ΣH_2S (Thamdrup and Canfield, 1996; Chapter 5.).

From the known amount of <S^0> and the average <NO_3^->/<S^0>-ratio in a given depth interval one can calculate the amount of NO_3^- being accumulated by *Thioploca* cells and transported into the sediment. If the <NO_3^-> were completely released from the cells, it would lead to porewater concentrations of about 1 mM (Fig. 9B). There is evidence in the published literature that some measurements of porewater NO_3^- were influenced by <NO_3^-> released from vacuolated sulfur bacteria during porewater sampling.

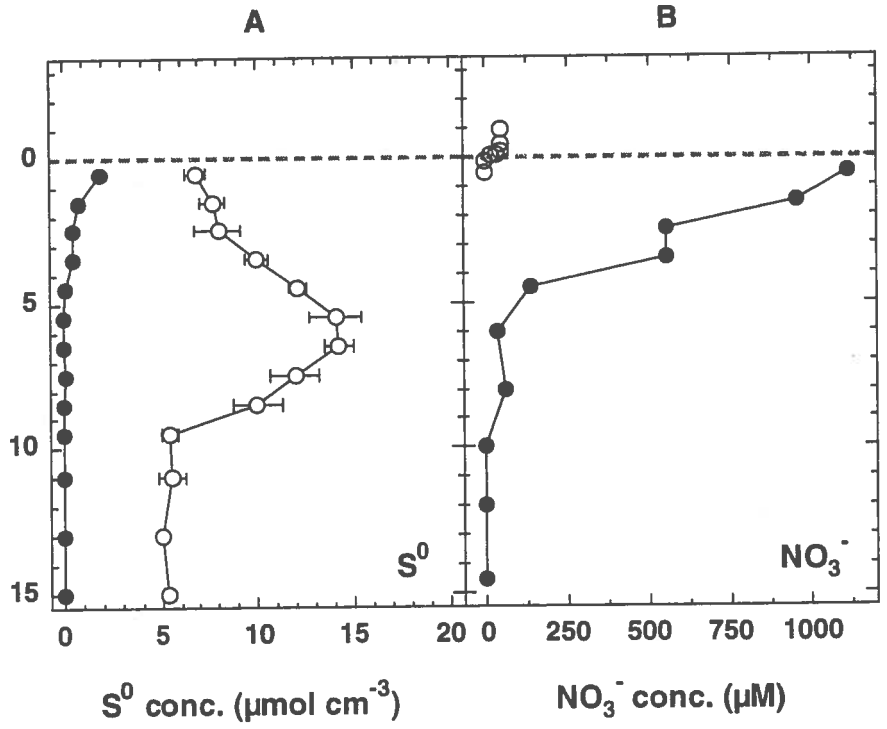

Fig. 9: Intracellular (closed symbols) and extracellular (open symbols) pools of S^0 and NO_3^- in sediment of Station 7. The broken lines indicate the sediment surface.

For instance, Henrichs and Farrington (1984) noted that the NO_3^- porewater concentrations in a *Thioploca*-containing sediment were higher than in the overlying seawater. In sediments from the Peru upwelling area NO_3^- was found in significant concentrations down to an unusual depth of 10 cm, and the maximum concentrations (up to 102 µM) were conspicuously high (Froelich et al., 1988). In later publications, where unusually high nitrate concentrations were observed, it was already suspected that they were affected by NO_3^- leaking from disrupted cells (Reimers et al., 1996; Thamdrup and Canfield, 1996).

Over the recent years a variety of approaches have been applied to estimate the contribution of *Thioploca* spp. to sulfide oxidation. Although the reported values vary from 3 to 91% (Ferdelman et al., 1997; Fossing et al., 1995; Otte et al., 1999; Thamdrup and Canfield, 1996), most estimates fall in the range of 20-30%. However, even if *Thioploca* spp. were not the dominant player in sulfide oxidation in these sediments they are most significant for the sedimentary nitrogen cycling. Organic matter in the sediments off Concepción Bay is almost exclusively degraded via sulfate reduction (Thamdrup and Canfield, 1996):

$$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 53\ SO_4^{2-} \Rightarrow 106\ HCO_3^- + 53\ HS^- + 16\ NH_4^+ + 40\ H^+ + PO_4^{3-} \quad (1)$$

$$13.3\ HS^- + 13.3\ NO_3^- + 13.3\ H_3O^+ \Rightarrow 13.3\ SO_4^{2-} + 13.3\ NH_4^+ \quad (2)$$

If only 25% of the produced sulfide was oxidized by *Thioploca* spp. according to Eq. 2 this would lead to an 83% increase of the sedimentary ammonium production. Since ammonium is not lost from the environment, in contrast to N_2, and since nitrogen is the limiting factor for phytoplankton growth in today's ocean (Codispoti, 1989), this form of nutrient regeneration could have considerable consequences for the primary productivity in the area.

Acknowledgments

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CHAPTER 5

Early Diagenesis and Isotope Biogeochemistry of Sulfur in *Thioploca*-Dominated Sediments off Chile

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Abstract

The biogeochemistry of sulfur was investigated in continental shelf sediments off central Chile which are dominated by filamentous sulfur bacteria, *Thioploca* spp. Dissolved and solid iron and sulfur species, including the sulfur intermediates sulfite, thiosulfate, and elemental sulfur were analyzed at high resolution down to 20 cm depth. All stations were characterized by high rates of sulfate reduction, but only the sediments within the Bay of Concepción contained significant amounts of hydrogen sulfide. Due to advection and/or in-situ reoxidation of ΣH_2S, porewater sulfate was mostly close to bottom water concentrations. Elemental sulfur was by far the dominant sulfur intermediate. Nitrate- and sulfur-storing bacteria were abundant, yet the major part of S^0 was extracellular. The concentrations of sulfite and thiosulfate were mostly in the submicromolar range. The distribution of sulfur species and dissolved iron indicated that the reaction of H_2S with FeOOH is an important pathway for sulfide oxidation and sulfur intermediate formation, which was in agreement with the stable sulfur isotope composition of co-existing elemental sulfur and iron monosulfides. For the bay station, the sulfur isotope data indicated that pyrite formation via reaction of FeS with polysulfides or H_2S is plausible. At the shelf stations, however, pyrite was significantly depleted in ^{34}S relative to the potential precursors AVS and S^0. Mass balance considerations suggest that deeper pyritization required a source of light hydrogen sulfide. The $\delta^{34}S$ values of FeS_2 were as low as –38 ‰ and are among the lightest values found yet in organic rich marine sediments.

1. Introduction

Sulfate reduction is the major pathway for the anaerobic mineralization of organic matter in coastal marine sediments (e.g. Jørgensen, 1982). In the sediments off central Chile, which are influenced by upwelling and are located in a nitrate-rich oxygen minimum zone (Fossing et al., 1995), organic matter is almost exclusively degraded via sulfate reduction (Thamdrup and Canfield, 1996). Despite the high rates of sulfate reduction, near surface sediments contain very little dissolved sulfide ($\Sigma H_2S \equiv H_2S + HS^- + S^{2-}$) and dissolved sulfate remains near seawater concentrations, suggesting efficient reoxidation of the reduced sulfur species (Ferdelman et al., 1997).

Although many studies were concerned with the chemistry and kinetics of abiotic ΣH_2S oxidation by oxygen, Mn(IV) or Fe(III) (Boulègue and Denis, 1983; Chen and Morris, 1972; Millero et al., 1987; Zhang and Millero, 1993; Burdige and Nealson, 1986; dos Santos and Stumm, 1992; Pyzik and Sommer, 1981; Yao and Millero, 1993; 1996), sulfide oxidation pathways and the regulation of these processes in continental shelf sediments are far from being

understood. *Chemical* oxidation of ΣH_2S does not primarily lead to sulfate, but passes through various intermediate oxidation states, e.g. polysulfides (S_n^{2-}), elemental sulfur (S^0), thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}). *Biologically*, ΣH_2S may be rapidly oxidized to sulfate in the presence of a suitable electron acceptor, such as oxygen or nitrate (Kuenen, 1989). Limited supply of these oxidants, however, may also lead to the biological formation of intermediate sulfur compounds (van den Ende and van Gemerden, 1993).

Sulfur intermediates are of general importance for the early diagenesis of carbon, iron and sulfur. For example, trace metal solubility and mobility are influenced by complexation with polysulfides and thiosulfate (Jacobs and Emerson, 1982; Morse et al., 1987). Polysulfides are also suspected to be involved in the formation of pyrite (Luther III, 1991), thiols (Vairavamurthy and Mopper, 1989) and organic polysulfides (Kohnen et al., 1989). Sulfonates have been proposed to be formed by the reaction of sulfite or thiosulfate with reactive organic matter (Vairavamurthy et al., 1994). Sulfur intermediates may further be oxidized to sulfate in the presence of a suitable electron acceptor, but may also be reduced to sulfide in the presence of metabolizable organic substrates (e.g. Kelly, 1989; Widdel, 1988). Some microorganisms can grow by the disproportionation of S^0, $S_2O_3^{2-}$ and SO_3^{2-}. In this case, the sulfur compound acts as both electron acceptor and donor and is thereby partly reduced to sulfide and partly oxidized to sulfate (Bak and Cypionka, 1987; Thamdrup et al., 1993). Large sulfur isotopic fractionations are observed during the bacterial disproportionation of sulfur intermediates in culture experiments (Canfield and Thamdrup, 1994; Cypionka et al., 1998; Habicht et al., 1998), and it has been suggested that this process may contribute to the observed strong enrichment of ^{32}S in natural sedimentary sulfides (Jørgensen, 1990; Canfield & Teske, 1996).

However, only few data are available on the distribution of SO_3^{2-}, $S_2O_3^{2-}$ and S^0 in marine sediments (Gagnon et al., 1996; Luther III et al., 1985; MacCrehan and Shea, 1995; Thamdrup et al., 1994a; Troelsen and Jørgensen, 1982). This is mostly due to the fact that the concentrations of SO_3^{2-} and $S_2O_3^{2-}$ in the environment are very low and that sufficiently sensitive analytical methods were lacking.

The aim of the present study was to investigate sulfide oxidation in upwelling influenced sediments with intense sulfate reduction but with low accumulation of dissolved sulfide. We present data on speciation and distribution of inorganic sulfur and iron compounds with special emphasis on the sulfur intermediates such as S^0, $S_2O_3^{2-}$ and SO_3^{2-}. Mechanisms of their formation and transformation and the influence of *Thioploca* on sulfur speciation are discussed, and the consequences of intense sulfur cycling on the stable sulfur isotope partitioning were studied at high depth resolution.

2. Study area

The continental shelf region off the Concepción Bay in central Chile is characterized by intense seasonal upwelling. Between austral late spring and early fall, southern and southwestern winds prevail and the northward flowing Subantarctic Surface Water is forced off the coast leading to upwelling of Equatorial Subsurface Water (ESSW) from the Poleward Undercurrent at 100 to 400 m (Strub et al., 1998). The ESSW is characterized by high salinity (34.4 - 34.8 ‰), low temperature (8.5 - 10.5 °C), low oxygen (< 20 µM) and high nitrate (about 25 µM) concentrations (Strub et al., 1998). Upwelling off central Chile is intermittent and usually lasts 2 to 7 days (Hill et al., 1998). The transport of deep, nutrient rich water up into the euphotic zone stimulates biological primary and secondary production and makes these areas the most productive systems of the world oceans with major ecological and economic significance. For the upwelling area off central Chile, a primary production of 9.6 g C m^2 d^{-1} has been reported (Fossing et al., 1995), which is one of the highest observed in marine environments. Despite this high primary productivity the sedimentation rate is only 1-2.2 mm a^{-1} (Thamdrup and Canfield, 1996).

The sediments off central Chile are populated by large sulfide-oxidizing bacteria of the genera *Thioploca* and *Beggiatoa* (Gallardo, 1977; Schulz et al., 1996; Schulz et al., 2000). Both have been found to accumulate nitrate in large vacuoles (Fossing et al., 1995; Chapter 4 this thesis). Physiological experiments with *Thioploca* spp. revealed that sulfide is first oxidized to intracellular elemental sulfur (<S^0>) and then further to sulfate, while nitrate is reduced to ammonium (Otte et al., 1999). Thus, the *Thioploca/Beggiatoa* community couples the biogeochemical cycles of nitrogen and sulfur. Reported estimates on the contribution of *Thioploca* to sulfide oxidation in surface sediments off the Concepción Bay vary between 6–91% (Fossing et. al., 1995; Thamdrup and Canfield, 1996; Ferdelman et al., 1997; Otte et al., 1999) of the SRR, but most values fall in the range of 20-30%.

3. Material and Methods

3.1 Sampling

During January and February 1997 we repeatedly collected sediment samples from four different stations in the Bay of Concepción and the adjoining shelf region (Fig. 1). Station 4 (36° 38' 08" S; 073° 02' 03" W) is 24 m deep and located within the bay. During the sampling period the sediment at this station was highly sulfidic and uniformly black, except for the uppermost 3-4 mm, which were brownish, presumably due to newly settled detrital phytoplankton. The top 4.5-5 cm of the sediment had a soupy consistence, and sheathless, filamentous sulfur bacteria were abundant in this layer.

The sediment at Station 7 (36° 36' 05" S; 073° 00' 06" W; 32 m deep) at the mouth of the bay, was more consolidated and without sulfide smell. The upper first 1.5 cm of the sediment had a spongy consistence, a brownish color, and was densely populated by filamentous sulfur bacteria (mainly *Thioploca* spp.). Below this layer was a 0.4-cm thick band of black iron sulfide followed by gray-brownish sediment underneath. Faunal burrows were observed. The sediments of the shelf Station 14 (36° 32' 01" S; 073° 03' 00" W; 64 m deep) and Station 18 (35° 30' 08" S; 073° 07' 06" W; 88 m deep) had a spongy layer of 0.5-1 cm overlying a thin black layer of 0.2-0.3 cm thickness and a gray sediment below. *Thioploca* spp. was much less abundant than at Station 7.

The same stations were revisited in March 1998 in order to study the effect of the El Niño-Southern Oscillation (ENSO) on the *Thioploca*-community and sulfate reduction rates. During this sampling also sediment cores for the determination of the stable isotopic composition of different sulfur species were collected.

El Niño situations in this area are characterized by increased surface water temperature, oxic conditions in the water column, limited nutrient availability and, hence, lower primary productivity (Arntz et al., 1991; Mann and Lazier, 1996). The measured sedimentary sulfate reduction rates were lower than typical for this season (B. Strotmann, unpubl.) and pore water sulfide concentrations were low at all stations. At Station 4, a maximal concentration of 70 µM was determined at 20 cm depth, and on the shelf stations ΣH_2S was constantly below 5 µM (B. Strotmann, unpubl.). The abundance of *Thioploca* spp. was greatly reduced (Schulz et al., 2000).

Sediment cores were recovered using a small Rumohr-gravity corer on board the R/V *Kay Kay* of the University of Concepción and stored at 4 °C until further sample processing was done in the shore-based laboratory in Dichato. Pore water was obtained by pressure filtration (0.45 µm Millipore filters) at room temperature in a N_2-filled glove bag and directly collected in 1.5 ml reaction tubes containing either 0.3 ml 2% $ZnCl_2$ solution for sulfate and sulfide measurements or the derivatization-mixture for thiosulfate and sulfite determination. The exact amount of sample (about 500 µl) in each vial was determined by weighing.

3.2 Analytical methods

Pore water constituents: Sulfate was determined by non-suppressed ion chromatography and conductivity detection (Ferdelman et al., 1997). Separation was done on an IC-Pack™ anion exchange column (50x4.6 mm; Waters) with a flow of 1 ml min^{-1} of 1 mM isophtalic acid in 10% methanol (pH 4.6, adjusted with saturated sodium borohydrate solution). Samples for sulfite (SO_3^{2-}) and thiosulfate ($S_2O_3^{2-}$) were derivatized at room temperature in the dark with a mixture of 40 µl monobromobimane (Sigma; 48 mM in acetonitrile) and 50 µl HEPES-EDTA buffer (pH 8, 500 mM, 50 mM) (Fahey and Newton, 1987). The derivatization

reaction was stopped after 30 min by adding 100 µl methanesulfonic acid (312 mM), then the samples were frozen at -20 °C until analysis within the next few days. A Sykam gradient controller S2000 combined with a LiChrosphere 60RP select B column (125x4 mm, 5 µm; Merck) and a Waters 470 scanning fluorescence detector (excitation at 380 nm; detection at 480 nm) was used for analysis. Eluent A was 2.5% (v/v) acetic acid pH 3.5 (adjusted with 5N NaOH), eluent B was 100% HPLC-grade methanol, and the flow rate was 1 ml min^{-1}. A modification of the gradient conditions described by Rethmeier et al. (1997) was used: start 10% B, 7 min 12% B, 15 to 19 min 30% B, 23 min 50% B, 30 min 100% B, 33 min 100% B, 34 min 10% B, 39 min 10% B, injection of the next sample. Separate standards for sulfite, thiosulfate and sulfide were prepared in degassed Milli-Q water in a N_2-filled glove bag. No difference was observed between calibration curves with standards prepared in seawater or in Milli-Q water. Detection limits for thiosulfate and sulfite were about 100 nM. The precision for repeated injection of 10 µM standards was better than ±3% SD. Dissolved sulfide was either determined on $ZnCl_2$-preserved pore water samples by the colorimetric methylene blue method of Cline (1969) or directly from unpreserved samples using the bimane method. Detection limit for the method was about 0.5 µM.

Solid phase sulfur: Sediment samples for solid phase sulfur species were sliced, fixed in zinc acetate (20 %) and stored in 50 ml polyethylene centrifuge tubes at -20 °C. Elemental sulfur (S^0) was extracted from the fixed sediment for 16 h on a rotary shaker with pure methanol; the sample to extractant ratio was about 1/20 (ww/vol). The amount of intracellular <S^0> was determined in one core from Station 7 by manually picking all visible filaments under a dissection microscope. Then <S^0> was extracted from the collected filaments with methanol and quantified by HPLC. A Sykam pump (S1100), a UV-VIS Detector (Sykam S3200), a Zorbax ODS-column (125x4 mm, 5 µm; Knauer, Germany) and 100% methanol (HPLC grade) at a flow rate of 1 ml per minute were employed. S_8 eluted after 3.5 minutes and was detected at 265 nm; the detection limit was about 1 µM, and the analytical precision of the method was ±0.5 % SD. Acid volatile sulfur (AVS ≡ ΣH_2S + FeS) and chromium reducible sulfur (CRS ≡ S^0 + FeS_2) were determined by a two-step distillation with cold 6 N HCL followed by boiling 1 M acidic $CrCl_2$ solution (Fossing and Jørgensen, 1989). The liberated H_2S was collected in Zn-acetate (20 %), traps and the concentration was determined using the colorimetric method after Cline (1969). The amount of pyrite was calculated by subtracting S^0 from the total CRS concentration. The precision for repeated distillation (n = 4) of the same sample was ±11 % for the AVS fraction and ±8 % for the CRS fraction.

Iron and manganese: Iron and manganese oxides were extracted with dithionite-citrate-acetic acid (DCA, pH 4.8) for 2 hours at room temperature (Thamdrup et al., 1994). Because dithionite interferes with the photometric iron determination, iron was quantified in aged (i.e. dithionite was oxidized) extracts with a hydroxylamine hydrochloride containing Ferrozine solution (Thamdrup et al., 1994). The dithionite treatment also extracts FeS; therefore the oxide

values were corrected by subtracting AVS. Manganese was determined in the DCA extracts by means of AAS. As reported by Thamdrup et al. (1994), manganese extracted by DCA gives somewhat lower values, but show the same trends as with the more common acidic ammonium oxalate extraction. Concentrations of dissolved iron and manganese were determined on filtered and $ZnCl_2$-preserved pore water samples using ICP-OES (Perkin Elmer Optima 3000 XL). Diluted samples were acidified prior to ICP measurements with HNO_3 (reagent grade), and blank contributions to the measured metal concentrations from the $ZnCl_2$ were considered.

3.3 Stable sulfur isotopes

Samples for ^{34}S stable isotope analysis were collected at the same stations as described above, in March 1998 during an ENSO event. At that time porewater ΣH_2S concentrations were low at all stations and, therefore, its stable isotopic composition could not be determined.

Zinc acetate-fixed samples were centrifuged at 5000 rpm for 5 minutes. The supernatant was removed and filtered through a GF/F filter (Gelman). Sulfate in the porewater was precipitated as $BaSO_4$ (Grasshoff et al., 1983). Elemental sulfur was extracted from the sediment pellet by adding N_2-gassed methanol. The closed samples were first treated in an ice-cooled ultrasonifier (2 x 15 min) and finally put on a rotary shaker for 6 h. Thereafter, the samples were centrifuged, and the supernatant was removed and filtered (GF/F). The dissolved elemental sulfur was reduced in a distillation line by adding acidic $CrCl_2$ solution and careful heating. The evolved H_2S was precipitated as Ag_2S in a $AgNO_3$-containing trap. The AVS fraction and pyrite sulfur were separated by the two step distillation as described above, but with $AgNO_3$ traps. The Ag_2S and $BaSO_4$ precipitates of the different fractions were filtered (0.45 µM, Millipore), washed with demineralized water, dried and weighed. The stable isotopic composition of Ag_2S and $BaSO_4$ powders was determined by means of combustion isotope ratio monitoring mass spectrometry (C-irmMS) with an elemental analyzer (Carlo Erba EA 1108) coupled to a Finnigan Mat 252 mass spectrometer via a Finnigan Mat Conflo II split interface (Böttcher et al., 1998a). $^{32}S/^{34}S$ ratios are given in the δ-notation relative to the Vienna-Canyon Diablo Troilite (V-CDT) standard. IAEA standards IAEA-S-1 and IAEA-S-2 were used to calibrate the mass spectrometer. $\delta^{34}S$ values of +20.6, -32.1, +17.3, and +16.3 ‰ were measured for reference materials NBS-127 ($BaSO_4$), IAEA-S-3 (Ag_2S), NBS-123 (ZnS), and IAEA-S-4 (S^0), respectively.

Table.1: Range and median values of biogeochemical parameters characterizing the 4. Medians were determined from all available values of a core.

	Station 4 Median (min/max)	Station 7 Median (min/max)	Station 14 Median (min/max)	Station 18 Median (min/max)
SRR (nmol $cm^{-3}d^{-1}$)[a]	389 (51/1169)	163 (29/603)	46 (31/116)	45 (21/258)
ΣH_2S (µM)	690 (433-1055)	1.0 (0.4/5.3)	0.2 (0/0.6)	0.4 (0/1.4)
SO_4^{2-} (mM)	23 (22/25)	26 (25-27)	27 (26/28)	26 (24/27)
SO_3^{2-} (µM)	0.9 (0.4-/2.6)	0.2 (0.1/0.5)	0.2 (0.2/0.4)	0.2 (0.1/0.3)
$S_2O_3^{2-}$ (µM)	2.7 (1.3/43)[b]	0.4 (0.2/1.3)	0.3 (0.2/0.9)	0.3 (0.2/2.2)
S^0 (µmol cm^{-3})	5.5 (2.9/9.3)	2.8 (1.7/9.6)	1.7 (0.7/4.6)	1.5 (0.5/7.4)
AVS (µmol cm^{-3})	13.3 (0/31.5)	8.5 (3.5/14.3)	9.1 (0.2/31.7)	3.7 (0.9/5.1)
FeS_2 (µmol cm^{-3})	66 (0/192)	111 (7/142)	38 (9/69)	58 (11/79)
Fe^{2+} (µM)	12 (4/20)	49 (12/103)	130 (24/244)	26 (10/174)
Mn^{2+} (µM)	7 (5/11)	8 (5/12)	5 (0/21)	7 (4/8)
$\delta^{34}SO_4^{2-}$ (‰)[c]	21.2 (20.6/22)	20.7 (20.4/20.8)	20.8 (20.4/22.1)	21.3 (20.7/23.1)
$\delta^{34}S^0$ (‰)[c]	-24.5 (-29.2/-21.2)	-30.8 (-33.3/-28.1)	-26.6 (-27.8/-24.8)	-24.7 (-30.6/-20.7)
$\delta^{34}S$ AVS (‰)[c]	-25.1 (-28.6/-15.1)	-28.2 (-29.5/-26.9)	-27.9 (-32.5/-23.6)	-27.7 (-31.3/-18.2)
$\delta^{34}S$ FeS_2 (‰)[c]	-28.8 (-30.2/-27.7)	-34.2 (-36.5/-32.2)	-31.6 (-36.4/-26.7)	-29.7 (-37.2/-27.9)

a) SRR: sulfate reduction rates, from B.Strotmann with permission; b) St. 4, calculated from the values below 3 cm.; c) $\delta^{34}S$-values in ‰ relative to the V-CDT standard.

4. Results and Discussion

4.1 Sulfate and iron reduction

Due to the lack of oxygen in the bottom waters, the high mineralization rates found in surface sediments are dominated by sulfate reduction (Thamdrup and Canfield, 1996). Although bacterial sulfate reduction rates (SRR) were high, up to 1170 nmol cm^{-3} d^{-1}, the concentrations of interstitial water sulfate remained nearly constant down to 20 cm depth (Fig. 2; Table 1). The SRR typically showed a near-surface maximum and decreased downcore and the mean SRR decreased with the distance from shore. These data are in agreement with previous measurements from 1994 (Ferdelman et al. 1997). The maximum SRR observed at the shelf Stations 14 and 18 are among the highest reports for typical coastal marine sediments (Skyring, 1987) or even exceed them (St. 7). The constant pore water sulfate agreed with a lack of ΣH_2S accumulation at the shelf stations. Although the highest SRR were observed in the top centimeters of the core, this was not reflected in the pore water concentrations of ΣH_2S that were mostly below 1 µM (Fig. 2A, B). High concentrations of dissolved iron in the near-surface pore water (Fig. 2D) indicate the presence of reducible ferric iron in the solid phase keeping the pore water sulfide concentrations low (Canfield, 1989). Bisulfide may react with Fe(III) oxides to form Fe^{2+}, iron sulfide and elemental sulfur (Pyzik and Sommer, 1981):

$$2FeOOH + HS^- + 5H^+ \Rightarrow 2Fe^{2+} + S^0 + 4H_2O \qquad (1)$$

$$Fe^{2+} + HS^- \Rightarrow FeS + H^+ \qquad (2)$$

The products of bacterial sulfate reduction should, therefore, not only be reflected by dissolved hydrogen sulfide, but also by the contents of acid volatile sulfides (AVS; mostly FeS) and elemental sulfur (see below). However, the concentrations of AVS are low at all shelf stations (Table 1, Fig. 2E), particularly close to the sediment surface, where most of the sulfide is produced. The highest concentrations of AVS were usually observed at intermediate depths suggesting that FeS is not only further oxidized in the near-surface sediment sections, but also consumed/transformed in the deeper parts. Anaerobic bacteria oxidizing FeS and Fe^{2+} with nitrate as electron acceptor have recently been described by Straub et al. (1996). The concentrations of dissolved manganese (Mn^{2+}) were mostly less than 10 µM (Table 1) and showed little variations with depth and between the different stations.

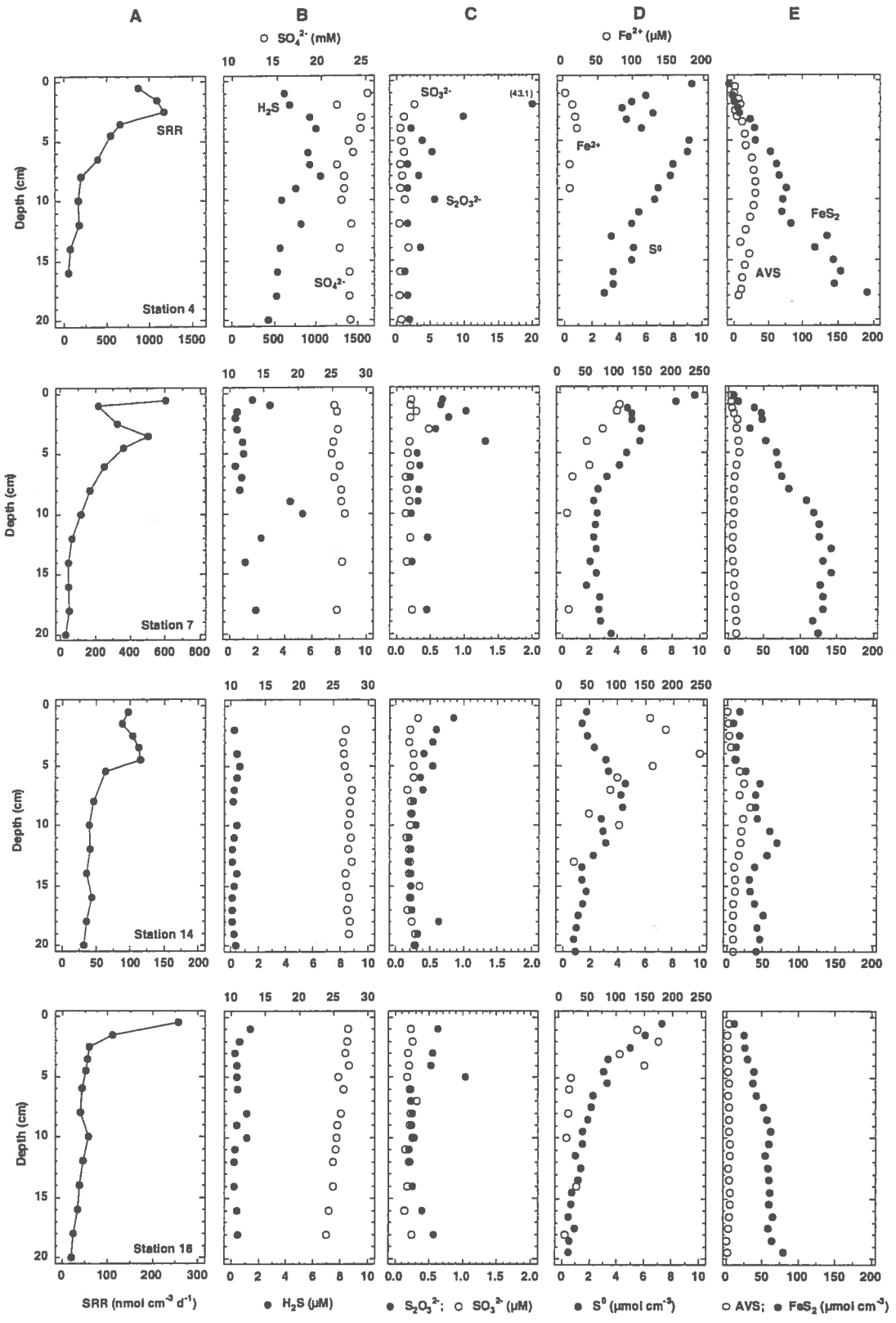

Figure 2: Sulfate reduction rates, solid phase and dissolved sulfur and iron speciation for all sampling stations. A) volumetric sulfate reduction rates, B) profiles of sulfate and sulfide, C) dissolved sulfur intermediates sulfite and thiosulfate, D) dissolved iron and solid phase S^0, E) solid phase sulfur AVS and FeS_2. Samples are from Jan./Feb. 1997.

SRR within the Bay of Concepción was up to 10 times higher than at the shelf stations (Table 1), likely due to upwelling-induced progressive eutrophication of the bay (Ahumada et al., 1983). Also here, pore water SO_4^{2-} decreased only slightly with depth, but ΣH_2S concentrations exceeded 1 mM (Table 1, Fig. 2B). Additionally, a significant flux of sulfide into the bottom water was indicated by the pore water profile. The high ΣH_2S and low Fe^{2+} concentrations show that the most reactive iron oxide pool was depleted (Canfield, 1989). Nevertheless, some dissolved iron was present even in 9 cm depth, which could indicate that iron oxide of lower reactivity was reduced by ΣH_2S and Fe^{2+} was released. However, since dissolved iron was determined as total iron in filtered pore waters, a minor contribution of colloidal or complexed ferric iron (Luther et al., 1996) cannot be ruled out completely.

The low concentrations of reduced sulfur species in spite of the high sulfate reduction rates suggest a fast reoxidation of ΣH_2S to sulfate. However, also intermediate oxidation products may form (e.g. Thamdrup et al., 1994; Troelsen and Jørgensen, 1982).

4.2 Sulfur intermediates

Depending on the electron donors (ΣH_2S, FeS, FeS_2), the electron acceptors (O_2, NO_3^- Fe(III), Mn(IV)), the ratio between the reactants, and the mode of the process (chemical, biological), different products are formed during sulfide oxidation (Table 2).

Thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}): The intermediate sulfur species sulfite and thiosulfate were detected at all stations and in all sediment depths. Sulfite concentrations were about 0.2 µM on the shelf and 0.9 µM in the bay (Table 1; Fig. 2C) and did not vary significantly with depth. Compared to the few previous studies, our values are in the same concentration range as determined by Thamdrup et al. (1994), but are much lower than in other reports. Boulègue et al. (1982) measured sulfite concentrations of up to 177 µM at the top of a salt marsh core, where intensive sulfide oxidation was taking place. Sulfite was below the detection limit of 0.1 µM in the deeper sediment sections. Sulfite concentrations determined in other marine sediments were between 7 µM and 71 µM (Luther III et al., 1985; Mopper and Taylor, 1986).

Although sulfite is observed in many sulfide oxidation reactions (Table 2), it does not accumulate due to its reactivity towards oxygen, elemental sulfur and sulfide. Under oxic conditions sulfite is rapidly oxidized to sulfate (Zhang and Millero, 1991), whereas in the presence of ΣH_2S or S^0 thiosulfate may form (Chen and Morris, 1972; Heunisch, 1977). Incubation experiments with ΣH_2S, $S_2O_3^{2-}$ or SO_3^{2-} additions to shelf sediments of the Black Sea revealed that SO_3^{2-} disappeared at the highest rate and was transformed mostly to $S_2O_3^{2-}$ (Zopfi, unpublished data). Thus, this transformation pathway is also of importance under the low reactant conditions in nature. Sulfite is a strong nucleophile and can react with organic

molecules to form sulfonates ($R\text{-}SO_3^-$), which have been recognized as a major class of organic sulfur compounds in marine sediments (Vairavamurthy et al., 1994; Vairavamurthy et al., 1995). A reactant half-life of about 5 min has been reported, indicating that the reaction between sulfite and organic molecules can be rather fast (Vairavamurthy et al., 1994).

The thiosulfate concentrations measured in the shelf sediments were mostly in the submicromolar range (Tab 1, Fig. 2C), yet they were higher than the sulfite concentrations. This is in contrast to a few earlier reports (Thamdrup et al. 1994; Zhang and Millero, 1993) where sulfite was found to be more abundant than thiosulfate. Higher thiosulfate than sulfite concentrations are commonly observed in environments like salt marshes (Boulègue et al., 1982; Luther III et al., 1988, Vetter et al., 1989; Luther III et al., 1985), subtidal marine sediments (Luther III et al., 1985) and the chemocline of stratified water columns (Jørgensen et al. 1979; Zopfi et al. 2001). The $S_2O_3^{2-}$ concentrations increased towards the sediment surface (Fig. 2C). Two alternative explanations can be given for this distribution. Firstly, the dissolved iron and thiosulfate distribution correlated strongly, which may suggest that not only elemental sulfur (Eq. 1) but also thiosulfate is formed by the oxidation of sulfide with Fe(III) oxides (e.g. Pyzik and Sommer, 1981; dos Santos and Stumm, 1992). Secondly, *Thioploca* may have produced thiosulfate during sulfide oxidation, since the distribution of *Thioploca* and $S_2O_3^{2-}$ correlate as well. Indeed, $S_2O_3^{2-}$ production has been observed in incubation experiments with purified *Thioploca* filaments after sulfide addition (Otte et al., 1999). As these experiments were not performed with pure cultures, and $S_2O_3^{2-}$ was also detected in control incubations, the significance of *Thioploca* for $S_2O_3^{2-}$ production remains uncertain. Thiosulfate can be used as substrate for growth by thiobacilli and filamentous sulfide oxidizing bacteria (Kelly, 1989; Nelson and Hagen, 1995), but the affinity towards $S_2O_3^{2-}$ varies significantly between different genera and strains (van den Ende, 1997). For *Thioploca* it was shown that sulfide was clearly preferred as substrate, and $S_2O_3^{2-}$ was metabolized only very slowly (Otte et al., 1999).

The highest thiosulfate concentration was found at Station 4, with a maximum of 43.1 µM. Sediments at this station were very fluffy and could easily be resuspended and oxidized whereby thiosulfate may have formed. The thiosulfate concentrations deeper at St. 4 were also higher than at the other stations, yet they are the lowest measured under such highly sulfidic conditions (Vetter et al., 1989). Even then, we cannot completely rule out that some thiosulfate was artificially produced due to rapid oxidation of sulfide (Zhang and Millero, 1993) or polysulfides (Chen and Morris, 1972; Steudel et al., 1986) by a minor contamination of pore waters by oxygen during sediment sampling.

So far, sulfur intermediates in upwelling-influenced sediments have only been measured by Boulègue and Denis (1983). In the nutrient-rich sediments of Walvis Bay of Namibia, which are comparable to our Station 4, they found thiosulfate concentrations ranging between 15 and 145 µM.

In the absence of bacteria, thiosulfate is chemically stable under pH neutral conditions (Millero, 1991) and is less reactive towards organic compounds than sulfite (Vairavamurthy et al., 1994). Hence, the very low concentrations in the sediments off the Bay of Concepción are most likely due to the activity of bacteria catalyzing oxidation, reduction or disproportionation reactions (Jørgensen, 1990; Jørgensen and Bak, 1991; Thamdrup et al., 1994a).

Table 2: Products of the chemical or biological oxidation of major reduced sulfur compounds in marine sediments. The order of products from the left to the right signifies their quantiatitve importance. Only results from studies conducted at circumneutral pH are compiled. Intermediates, which are unstable under the experimental conditions or which are only observed in trace quantities are given in *Italics*. For experimental details we refer to the original literature.

S-species	Oxidant	Products	Comments [c]	Reference
H_2S	O_2	SO_4^{2-}, $S_2O_3^{2-}$, *SO_3^{2-}*	C	Zhang & Millero, 1993
	O_2	SO_4^{2-} $S_2O_3^{2-}$, *S_n^{2-}*, S^0	C	Chen & Morris, 1972
	O_2	SO_4^{2-}, *$S_2O_3^{2-}$*, *SO_3^{2-}*	M	Kelly, 1987
	O_2	S^0, $S_2O_3^{2-}$, SO_4^{2-}, *$S_nO_6^{2-}$*	M	van Ende & van Gemerden, 1997
	NO_3^-	S^0, SO_4^{2-}	S	Elsgaard & Jørgensen, 1992
	NO_3^-	S^0, SO_4^{2-}	M	Otte et al., 1999
	Mn_{IV}	S^0, $S_2O_3^{2-}$, SO_4^{2-}, *SO_3^{2-}*	C	Yao & Millero, 1996
	Fe_{III}	S^0, $S_2O_3^{2-}$, *SO_3^{2-}*	C	Pyzik & Sommer, 1982
S_n^{2-}	O_2	$S_2O_3^{2-}$, S^0	C	Steudel et al., 1986
FeS	O_2	S^0, $S_nO_6^{2-}$, $S_2O_3^{2-}$, SO_4^{2-}	C	von Rège, 1999
	NO_3^-	SO_4^{2-}	M	Straub et al., 1996; (pers. comm.)
	Mn_{IV}	SO_4^{2-} [a]	S	Aller & Rude, 1986
	Fe_{III}	SO_4^{2-} [a, b]	S	Aller & Rude, 1986
FeS_2	O_2	SO_4^{2-}, $S_nO_6^{2-}$, $S_2O_3^{2-}$	C	Moses et al., 1987
	Fe_{III}	SO_4^{2-}	C	Moses et al., 1987

a) no S-intermediates determined; b) only weak sulfate production; c) Type of study: C = chemical, M = microbiological, S = sediment incubation

Elemental sulfur (S^0): Zero-valent sulfur concentrations were generally 1-10 μmol cm^{-3}, and reached up to 14 μmol cm^{-3} (St. 7; Fig. 3A), which is in the upper range for marine sediments (Canfield and Thamdrup, 1996; Fossing, 1990; Gagnon et al., 1995; Thamdrup et al., 1994b; Thode-Andersen and Jørgensen, 1989; Troelsen and Jørgensen, 1982).

All stations were densely populated by filamentous sulfur bacteria, *Beggiatoa* or *Thioploca*. These organisms oxidize ΣH_2S to S^0 and further to SO_4^{2-}, using nitrate, which is thereby reduced to ammonium (Otte et al., 1999). Elemental sulfur is transiently stored in the cells in the form of bis-organylsulfanes, $R\text{-}S_n\text{-}R$ (Prange et al., 1999). Consequently, two forms of zerovalent sulfur can be differentiated in these sediments, which also reflect two different formation mechanisms.

Intracellular sulfur (<S^0>) contributed up to 27% of total S^0 in the sediment of Station 7 (Fig. 3) and showed a different depth distribution than extracellular S^0. Concentrations of <S^0> were highest at the sediment surface and decreased gradually with depth (Fig. 3), corresponding the distribution of *Thioploca* in the sediment (Schulz et al., 1996). If we assume that the S^0 oxidation rates are the same in filaments from all depths, then the oxidation rate of sulfide by *Thioploca* should be most effective at the sediment surface (Table 3).

Most of the S^0 in the sediments was extracellular with maximum concentrations found between 3 and 8 cm depth (Fig. 2 and 4). The distribution of S^0 and thiosulfate indicates that sulfide oxidation predominantly took place in the top 5 to 7 cm of the sediment. This is consistent with the apparent bioturbation horizon determined by the excess ^{210}Pb profiles (T. Ferdelman, unpublished) and the presence of dissolved iron originating from the reduction of detrital iron oxides by ΣH_2S. A subsurface maximum of S^0 may be found in a variety of coastal marine sediments (Troelsen and Jørgensen, 1982, Thamdrup et al., 1994, Zopfi unpubl.). Above the maximum, S^0 may be oxidized to sulfate, because the electron acceptors, O_2, NO_3^- and Mn(IV), may be supplied by bioturbation (Aller and Rude, 1988) or advection (Huettel et al., 1998). So far, advective transport of particulate metal oxides has only been described for sandy sediments but, since the *Thioploca*-mats in the top cm of the sediments are extremely porous (Reimers, 1982), could take place in these sediments. Faunal burrows have also been observed at all stations outside the bay. On the shelf, the benthic macrofauna is dominated by polychaetes (85% of total individuals) and small crustaceans (14%) (Gallardo et al., 1995).

Below the maximum, S^0 may be consumed by heterotrophic sulfur reducing (Widdel, 1988) or disproportionating bacteria (Thamdrup et al., 1993), or it may be incorporated into pyrite (Rickard et al., 1995; Wilkin and Barnes, 1996) or into organic matter via polysulfides (Mossmann et al., 1991).

In sediments from sulfidic environments (e.g. sulfureta, euxinic basins) maximum S^0 concentrations are usually observed at the sediment surface (Thode-Andersen and Jørgensen, 1989; Troelsen and Jørgensen, 1982; Zopfi, unpubl.). Although we expected a similar

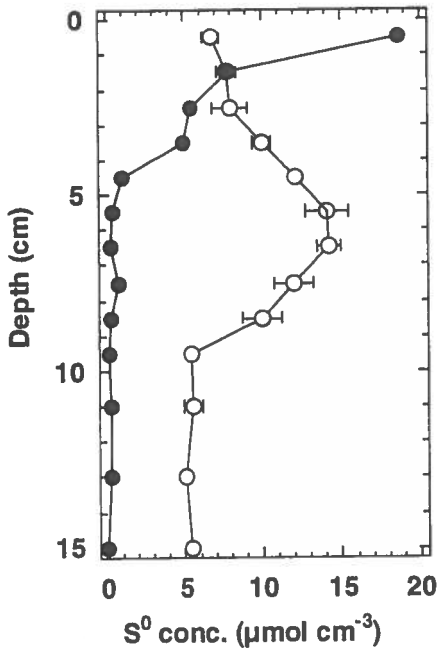

Figure 3: Distribution of S^0 in the bulk sediment (open circles) and in *Thioploca* filaments (closed circles) of Station 7. The concentrations for intracellular S^0 are multiplied by 10.

distribution at St. 4, this was clearly not the case. However, the pronounced seasonality observed at this station, showed that, whereas the sediment was highly sulfidic during the upwelling season, pore water sulfide concentrations were reduced to a few µM in austral winter (B. Strotmann, unpubl.). At the time of sampling, the S^0 distribution at St. 4 had probably not changed yet to the upwelling situation. Sedimentary S^0 represents a dynamic pool, but it is apparently not as dynamic as pore water sulfide.

Although the absence of free sulfide at St. 7, 14, and 18 and the distribution of the sulfur intermediates may be due to the reaction of sulfide with Fe(III) oxides, the constant sulfate concentrations still need to be explained. Chemical (Yao and Millero, 1996), microbiological (Lovley and Phillips, 1994), and sediment incubation experiments (Aller and Rude, 1988; Elsgaard and Jørgensen, 1992; King, 1990; Yao and Millero, 1996) have shown that Fe(III), in contrast to Mn(IV), is a rather inefficient oxidant for the complete oxidation of sulfides to sulfate (Table 2). Elemental sulfur is the main product of the non-enzymatic oxidation of sulfide with Fe(III) oxides (Table 2). The further transformation of S^0 to sulfate requires bacterial catalysis. Whereas bacteria that oxidize S^0 with Mn(IV) have been identified (Lovley and Phillips, 1994), attempts to enrich and isolate S^0 oxidizing Fe(III)-reducing bacteria have failed and have instead yielded S^0 disproportionating organisms (Thamdrup et al., 1993).

Table 3: Estimates of the contribution of *Thioploca* spp. to sulfide oxidation in sediments of Station 7 (February, 1997). Two different methods were used to determine the *Thioploca* biovolume and to illustrate the dependence of the sulfide oxidation rate calculations towards this parameter.

Depth	SRR [a]	<S^0>	<S^0>	Thioploca	Sulfide oxidation rate [e]		Thioploca	Sulfide oxidation rate [e]	
cm	nmol cm^{-3} d^{-1}	µmol cm^{-3}	µmol mm^{-3}	mm^3 cm^{-3}	nmol cm^{-3} d^{-1}	% of SRR	mm^3 cm^{-3}	nmol cm^{-3} d^{-1}	% of SRR
0-1	603	1.87	0.211 (8)	8.86	670	111.1	2.54	192	31.8
1-2	217	0.80	0.117 (8)	6.84	517	238.2	0.64	48	22.1
2-3	324	0.55	0.141 (9)	3.90	295	91.0	0.47	36	11.1
3-4	501	0.50	0.111 (7)	4.50	340	67.9	0.33	25	5.0
4-5	360	0.11	0.139 (*)	0.79	60	16.7	0.22	17	4.7
5-7	250	0.03		0.22	17	6.8	0.07	5	2
7-9	163	0.06	0.148 (8)	0.40	30	18.4	0.03	2	1.2
9-11	113	0.03	0.533 (12)	0.06	5	4.4	0.003	0.2	0.2
11-13	61	0.03		0.06	5	8.2	n.d.	n.d.	n.d.
13-15	45	0.02	0.669 (9)	0.03	2	4.4	n.d.	n.d.	n.d.
15-17	42	0.01		0.03	2	4.8	n.d.	n.d.	n.d.
Σ	2679				1943	72.5		325.2	12.1

a) Sulfate reduction rate determined by the $^{35}SO_4^{2-}$ radiotracer method. Data from B. Strotmann, with permission. b) Intracellular sulfur per volume sediment. c) Mean intracellular S^0 concentration in *Thioploca* trichomes collected at different depths. Number of measurements in brackets. *) Interpolated value. d) Biovolume calculated by dividing *b* by *c*. e) Calculated by multiplying the *Thioploca* biovolume with the specific sulfide oxidation rate of 75.6 nmol mm^{-3} d^{-1} taken from Otte et al. (1999). f) Biovolume determined by randomly picking 5 sheaths per depth interval. The number and biovolume of *Thioploca* in sheaths were determined by measuring length and diameter of the trichomes. This average value was multiplied by the total number of sheaths in the depth interval (Schulz et al., 1996). Data from H. Schulz, with permission. .

As the concentration of dissolved and solid phase manganese was low at all stations, a significant production of sulfate by Mn(IV) reduction seems unlikely. However, sulfate may be produced anoxically by *Thioploca* from $<S^0>$ (Eq. 3) or by disproportionating bacteria from sulfur intermediates (Eq. 4).

$$4\ S^0 + 3\ NO_3^- + 7\ H_2O \quad \Rightarrow \quad 4\ SO_4^{2-} + 3\ NH_4^+ + 2H^+ \qquad (3)$$

$$4\ S^0 + 4\ H_2O \quad \Rightarrow \quad SO_4^{2-} + 3\ HS^- + 5\ H^+ \qquad (4)$$

The potential for *Thioploca* to completely oxidize the produced sulfide and hence prevent sulfate depletion decreases with depth (Table 3). Below the surface layer, *Thioploca* can only account for a few percent of sulfate production. Although the quantitative importance of sulfur disproportionation in marine sediments is not known, mass and redox balance considerations show that this process cannot compensate the sulfate depletion due to the sulfate reduction. We propose instead that increased pore water exchange due to the high porosity and the activity of sediment dwelling organisms contributes to the constancy in pore water sulfate profiles.

4.3 Pyrite

Pyrite is the most abundant inorganic sulfur compound in marine sediments (e.g., Morse et al., 1987; Ohmoto et al., 1990). Iron monosulfide (AVS), on the other hand, is a metastable phase and will ultimately be transformed into pyrite (e.g., Morse et al., 1987; Rickard, 1997). The depth profiles of sedimentary iron sulfides show only minor AVS contents and a decrease of the pyrite contents from St. 4 to St. 18 (Fig. 2). In order to identify the factors controlling pyrite formation in the shelf surface sediments, we determined the degree of pyritization (DOP) according to:

$$DOP = Fe_{Pyritic} \ / \ (Fe_{Pyritic} + Fe_{DCA\text{-}soluble}) \qquad (5)$$

As suggested by Raiswell et al. (1994), the DOP was defined with respect to dithionite extractable iron. The dithionite procedure tends to result in lower iron concentrations than the more common HCl extraction because silicate-bound iron is not extracted (Raiswell et al., 1994). Consequently, DOP values according to Eq. 5 are slightly higher than those estimated from Fe_{HCl}. For St. 4, a DOP of 0.69 ± 0.04 was determined for depths below 2.5 cm (Fig. 4), This lies well within the range for semi-euxinic sediments (0.46 to 0.8; Raiswell et al., 1988).

The mean DOP determined for St. 18 was 0.45 ± 0.04 and lies between the values for oxic and for semi-euxinic sediments (Raiswell, 1988). This value suggests that iron is not the limiting factor for pyrite formation, since only about half of the reactive iron was transformed to pyrite. Two major factors can explain the low abundance of pyrite in the shelf sediments. Firstly, pyrite formation may be limited by the low ΣH_2S (and FeS) concentrations caused by oxidation and pore water exchange. As a second reason one must consider, that accumulation of sedimentary pyrite is controlled by a superimposition of FeS_2 precipitation and continuous reoxidation. Under anoxic conditions FeS_2 can be oxidized by Fe(III) (Moses et al, 1987), which may be produced by nitrate reducing Fe^{2+} or FeS oxidizing bacteria (Benz et al., 1998; Straub et al., 1996). Our results on the distribution of FeS and FeS_2 are in general agreement with measurements carried out in March 1994 at the same locations (Ferdelman et al., 1997). To gain further information on the formation of pyrite from potential precursor compounds, stable sulfur isotope measurements have been made on different sedimentary sulfur compounds in high depth resolution.

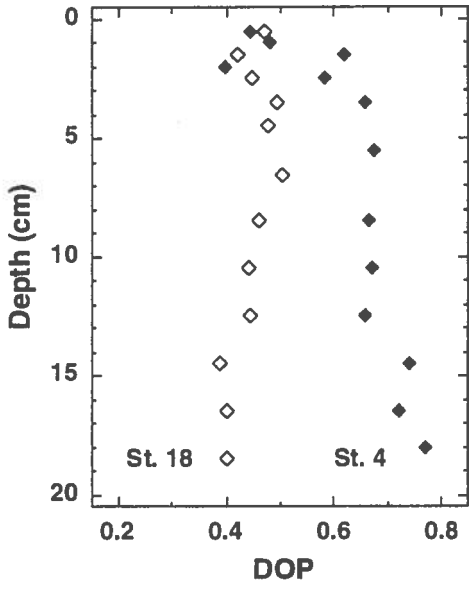

Figure 4: Degree of pyritization (DOP) vs. depth for St. 4 and St. 18

4.3 Stable sulfur isotopes

Pore water sulfate: At all stations the $\delta^{34}S$ of pore water sulfate within the upper 15 cm of the sediments varied only slightly between 20.4 and 21.3‰ (Fig. 5), which is similar to the isotopic composition of sea water sulfate ($\delta^{34}S \approx 20.5‰$). During bacterial dissimilatory reduction of sulfate the lighter ^{32}S isotope is preferentially reduced and, the residual sulfate becomes enriched in ^{34}S (e.g., Kaplan and Rittenberg, 1964). The similarity of the isotopic

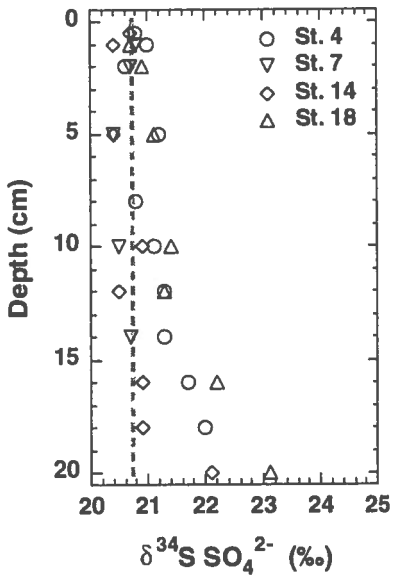

Figure 5: Stable isotopic composition of porewater sulfate of all stations. The broken line indicates the isotopic composition of seawater sulfate 20.6‰.

composition of interstitial water sulfate in the upper 15 cm with that of the bottom water, therefore, indicates either a rapid sulfate exchange between sea water and sediment by advection and bioturbation or a rapid in situ reoxidation of hydrogen sulfide to sulfate. Below 15 cm depth sulfate was slightly enriched in ^{34}S, up to 23‰ vs. V-CDT (Fig. 5; St. 18). Although at all stations the sulfate reduction rates were much higher one year earlier under non-ENSO conditions, similar $\delta^{34}S$ values for porewater SO_4^{2-} are anticipated based on the results from the SO_4^{2-} determinations (Fig. 2).

Iron monosulfide and elemental sulfur: Pure culture studies have revealed that dissolved sulfide produced during bacterial dissimilatory sulfate reduction is typically depleted in ^{34}S by 10-25 ‰ (maximum 46 ‰) compared to precursor sulfate (Chambers and Trudinger, 1979). Since essentially open system conditions with respect to sulfate are inferred from the isotope data and the concentration measurements, the $\delta^{34}S$ of hydrogen sulfide should show the kinetic sulfur isotope effect during sulfate reduction (e.g., Kaplan and Rittenberg, 1964; Chambers and Trudinger, 1979). The ΣH_2S concentrations in the porewater were too low to measure the isotopic composition directly but AVS should closely reflect the isotopic composition of its precursor, hydrogen sulfide. Only a small fractionation in the range -0.5 to +1.2 ‰ was observed experimentally between metal sulfide precipitates and dissolved sulfide (Böttcher et al., 1998b).

AVS and elemental sulfur were depleted 38-55 ‰ in ^{34}S compared to coexisting pore water sulfate (Fig. 5). These two solid phases showed isotopic differences close to the sediment surface, but a few centimeters below the surface the $\delta^{34}S$ of AVS and S^0 agreed within a few per mil (Fig. 5). With respect to the isotopic composition of AVS in the uppermost cm, the two shelf stations differed from St. 4 and 7. At Station 14 and 18, AVS was the lightest sulfur pool in the top centimeters of the cores, and below 5 cm (St. 14) and 3 cm (St. 18) the isotopic compositions of AVS and S^0 were within <2 ‰ almost identical. This can either be due to isotopic exchange between the two phases or indicates a common origin of formation.

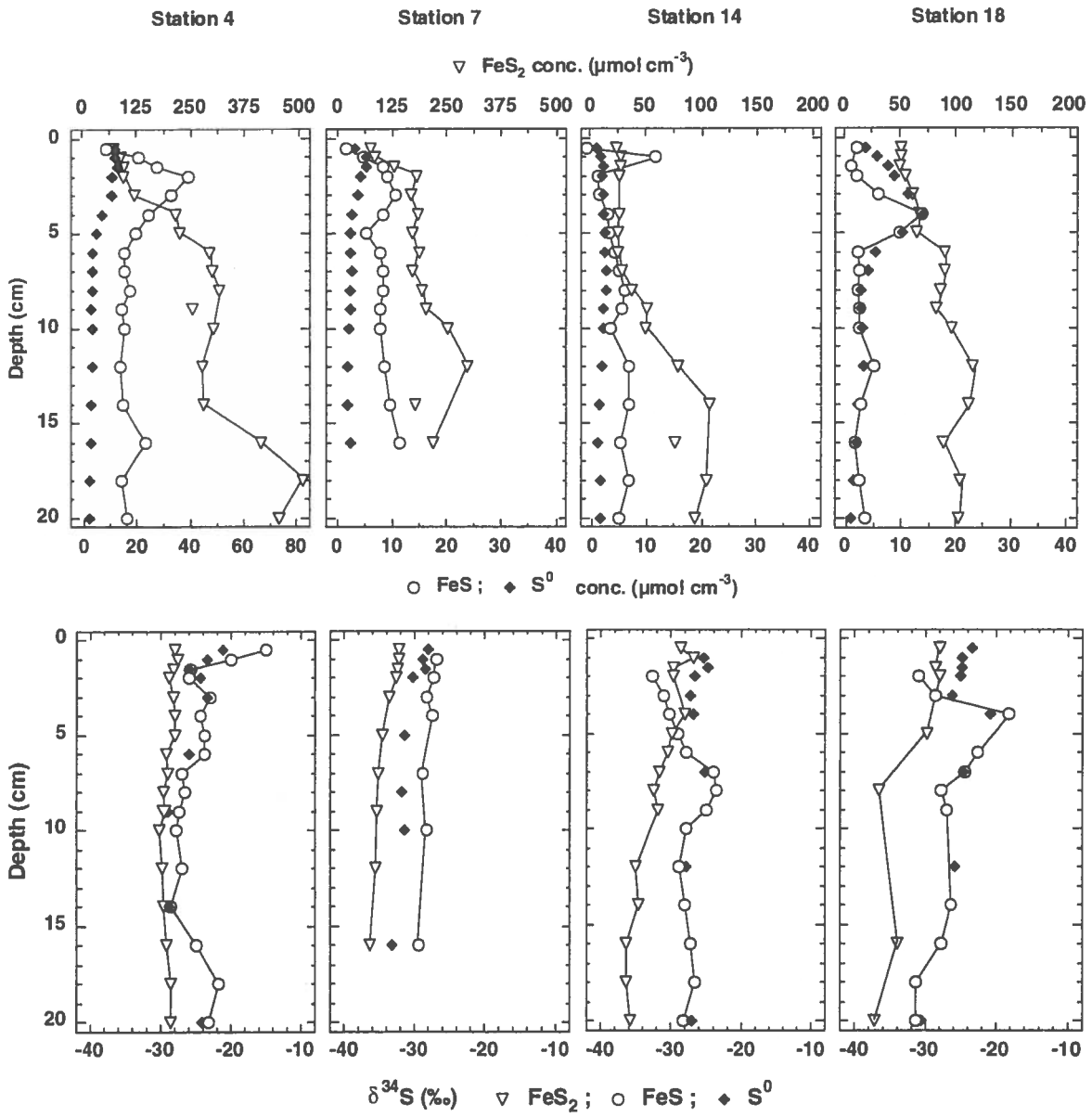

Figure 6: Distribution of solid phase sulfur compounds (top) and stable sulfur isotopic composition of FeS_2, AVS, and S^0 (bottom). All samples are from March 1998.

Under isotope exchange equilibrium conditions, elemental sulfur should be slightly enriched in ^{34}S compared to dissolved HS^-, but depleted in the heavy isotope compared to AVS (Sakai, 1968; Ohmoto and Goldhaber, 1997). Reactions leading to an exchange of sulfur isotopes between the reduced sulfur species ΣH_2S, S_n^{2-}, S^0 and FeS have been shown in anoxic marine sediments using ^{35}S radiotracers (Fossing et al, 1992). These reactions should be restricted at Stations 7, 14, and 18 due to the low ΣH_2S concentrations. At Station 4, however, minor isotope exchange between S^0 and AVS cannot be ruled out, because these sediments are highly sulfidic during part of the year. In agreement with the results from the S^0 and Fe^{2+} measurements, we propose that S^0 and AVS are produced by the reaction of ΣH_2S with detrital iron oxides (Eq. 1). This pathway also implies that the $\delta^{34}S$ value of ΣH_2S should be within a few per mil of S^0 and AVS. Under the assumption that no additional fractionation during sulfide oxidation took place, the pore water ΣH_2S of Station 4 should be depleted by a maximum of –49 ‰ relative to seawater sulfate and by about –53 ‰ at the other stations. Both values are slightly higher than the maximum fractionation of –46 ‰ observed in pure cultures (Kaplan and Rittenberg, 1964) and –43 ‰ that were observed in closed incubations of natural sediments (Habicht & Canfield, 1997).

Pyrite: Pyrite was the quantitatively dominant sulfur fraction in the deeper sections of all investigated sediments. In the majority of the sediment samples, pyrite was isotopically much lighter than coexisting AVS or elemental sulfur (Fig. 6). With the exception of Station 4, the pyrite fraction became increasingly enriched in ^{32}S with depth. Isotope fractionation between pore water sulfate and pyrite of up to –58 ‰ was observed in the sediments recovered in 1998 (Fig. 5). The isotope results are within the range observed for other marine sediments (Ohmoto et al., 1990).

Pyrite formation is often described as the reaction of zerovalent sulfur-containing compounds (S^0 or S_n^{2-}) and iron sulfide (e.g., Rickard et al., 1995):

$$FeS + S^0 \Rightarrow FeS_2 \qquad (6)$$

If no isotope fractionation is involved upon consumption of the educts, the isotopic composition of pyrite should be intermediate between the FeS and S^0. Isotopic results are in agreement with this assumption only in the uppermost few centimeters at Station 14 and 18 (Fig. 5). Deeper in the sediment and at all depths of Station 4 and 7, pyrite is enriched in ^{32}S compared to both AVS and elemental sulfur. However, pyrite formation may not only take place as suggested in Eq. 6, but H_2S can directly react with FeS to form pyrite (Rickard, 1997):

$$FeS + H_2S \Rightarrow FeS_2 + H_2 \quad (7)$$

Chemical and microbiological experiments have shown only a minor isotope effect during pyrite formation and, thus, that the isotopic composition of pyrite reflects the mixture of the sulfur sources (e.g. Sweeney and Kaplan, 1973; Price and Shieh, 1979; Canfield et al., 1998; Wilkin and Barnes, 1996). If we assume steady depositional conditions, mass balance calculations can be made to estimate the average isotopic composition of sulfur added to the pyrite pool at a given depth.

$$\delta^{34}S_{added} = \{ \delta^{34}S(FeS_2)_{z2} \cdot c(FeS_2)_{z2} - \delta^{34}S(FeS_2)_{z1} \cdot c(FeS_2)_{z1} \} / \{ c(FeS_2)_{z2} - c(FeS_2)_{z1} \}$$

Where $\delta^{34}S_{added}$ is the average isotopic composition of the added sulfur, $\delta^{34}S(FeS_2)$ is the isotopic composition of pyrite and $c(FeS_2)$ is the content of pyrite at depth *z1* and *z2*, respectively. According to this calculation (Table 4), a source of very light sulfur with $\delta^{34}S$ values between –29 ‰ and –45 ‰ (corresponding to a fractionation relative to sulfate of –50 ‰ to –65 ‰) is required to explain the isotopic composition of "deep" pyrite at St. 7, 14, and 18.

Table 4: Isotope mass balance calculations for pyrite formed over the depth interval z1 to z2.

Station	Depth intervall cm		Conc. FeS_2 µmol cm^{-3}		$\delta^{34}S$ FeS_2 ‰		$\delta^{34}S$ added ‰
#	z1	z2	z1	z2	z1	z2	
4	1.5	20	96.0	453.0	-28.2	-28.6	-28.7
7	1	16	84.7	220.4	-32.3	-36.5	-39.1
14	1.5	18	27.5	104.1	-29.9	-36.3	-38.6
18	1.5	20	49.3	103.0	-28.6	-37.2	-45.1

Disproportionation of sulfur intermediates: The sulfur isotope difference between pore water sulfate and reduced sulfur species is too large to be explained alone by the fractionation during bacterial sulfate reduction. The isotope effects associated with chemical or

microbial oxidation of hydrogen sulfide are small (Fry et al., 1986, 1988). On the other hand, the reduction of the sulfur intermediates thiosulfate and sulfite is associated with significant isotope discrimination (Kaplan and Rittenberg, 1964; Smock et al., 1998; Habicht et al., 1998), but the concentrations in the investigated sediments are too small to contribute to the overall sulfur isotope effect. Because the kinetic isotope effect associated with bacterial reduction of S^0 is only about –3 ‰ (Kaplan and Rittenberg, 1964), also this process should not be significant for to the overall isotope effect. A large enrichment has been observed during bacterial disproportionation of elemental sulfur, thiosulfate, and sulfite (Canfield et al., 1998; Cypionka et al., 1998; Habicht et al., 1998) and it was argued that these processes significantly contribute to the strong enrichment of ^{32}S in found sedimentary sulfides (Jørgensen, 1990; Canfield and Thamdrup, 1994).

The isotope difference between the $\delta^{34}S_{added}$ resulting from the mass balance calculations according to Eq. 8 and coexisting S^0 is between –6 ‰ to –15 ‰ for Station 7, 14, and 18. This is within the range found in experiments with disproportionating bacteria (Canfield et al., 1998; Cypionka et al., 1998; Habicht et al., 1998). Therefore, a one-step sulfur isotope fractionation associated with S^0 disproportionation is sufficient to produce sulfide with an isotope signature necessary to explain the variations in $\delta^{34}S$ values of pyrite at these stations. This requires, however, that all of the newly formed pyrite received ΣH_2S only from the disproportionating process, which is acting as the final step in a chain reaction with sulfate reduction and ΣH_2S oxidation. Actually, a continuous mixture of ΣH_2S from different metabolic pathways is expected to contribute to pyritization. Therefore, a repeated oxidation-disproportionation cycle as proposed by Canfield and Thamdrup (1994) may have to be considered. A superimposition of the isotope effects during oxidation and disproportionation is responsible for the significant correlation between isotope fractionation and the degree of sulfide reoxidation in recent marine sediments (Canfield and Teske, 1996). The upwelling area off the Concepción Bay fits into the proposed empirical relationship.

Station 4 had higher SRR than the other stations and high concentrations of pore water ΣH_2S (> 1 mM) during upwelling seasons. Under such conditions, ΣH_2S and S^0 form polysulfides (Eq. 9), which may further interact with FeS to form pyrite (Luther, 1991) according to:

$$HS^- + (n\text{-}1)S^0 \quad \Leftrightarrow \quad S_n^{2-} + H^+ \qquad (9)$$

$$FeS + S_n^{2-} \quad \Rightarrow \quad FeS_2 + S_{n-1}^{2-} \qquad (10)$$

The high concentrations of dissolved sulfide, on the other hand, hinder growth of disproportionating bacteria (Thamdrup et al., 1993) during most of the year and minimize the

addition of light sulfide to the FeS_2 pool. This is consistent with the mass balance calculations (Eq. 8) and the observation that pyrite at Station 4 is by at least 7 ‰ heavier than at the other stations. The fact that pyrite is still somewhat lighter than FeS and S^0, respectively, may be explained either by additional fractionation during pyrite formation or by the seasonal activity of disproportionating bacteria during austral winter when the pore water concentration of dissolved sulfide is transiently low (B. Strotmann, unpubl.).

5. Summary and Conclusions

The sulfur biogeochemistry was investigated at four stations in the upwelling area on the continental shelf of central Chile and within the Bay of Concepción. All stations were characterized by high rates of sulfate reduction, but only the sediment within the bay contained significant amounts of hydrogen sulfide. The pore water from all other stations was characterized to be suboxic by dissolved iron and manganese concentrations, and the absence of dissolved sulfide.

Dissolved and solid sulfur species indicated that reactions in the sediment took place under open system conditions with respect to sulfate, due to either advective transport and/or in-situ reoxidation of hydrogen sulfide. Besides sulfate also sulfur species with intermediate oxidation state were found as oxidation products. Elemental sulfur was the most abundant sulfur intermediate, but the concentrations were only modestly higher than in other coastal marine sediments. Although nitrate- and sulfur-storing bacteria were abundant in these sediments, the major part of S^0 was extracellular. The concentrations of sulfite were invariably low (about 0.2 µM). Thiosulfate concentrations were also mostly in the submicromolar range and were highest at the sediment water interface. The distribution of sulfur species and dissolved iron in the pore water suggested that the reaction of ΣH_2S with Fe(III) may be a major pathway for sulfide oxidation and sulfur intermediate formation. This reaction pathway is also in agreement with the stable sulfur isotope composition found in coexisting S^0 and iron monosulfides (AVS).

For the station within the Bay of Concepción, the sulfur isotope data indicate that pyrite formation via the polysulfide or the H_2S mechanism is possible. At the other stations, pyrite was significantly depleted in ^{34}S relative to the potential precursors AVS and S^0. Mass balance considerations suggested that deeper pyritization required a source of very light sulfide. So far, disproportionation of sulfur intermediates is the only known process producing sufficiently light sulfide to fulfill the mass balance requirements. Our results clearly show that open condition for sulfate and intense sulfur cycling can lead to solid sulfur species highly depleted in ^{34}S despite of high volumetric sulfate reduction rates. FeS_2, for instance, was depleted in ^{34}S up to -58 ‰ relative to seawater sulfate, being among the highest sulfur isotope fractionations observed in coastal marine sediments so far.

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CHAPTER 6

Summary

Comparative Measurements of Sulfur Intermediates

Due to a lack of suitable methods, knowledge about the distribution of the sulfur intermediates sulfite (SO_3^{2-}) and thiosulfate ($S_2O_3^{2-}$) in the environment is scarce and some reported values are affected problematic. Therefore, a method for the determination of SO_3^{2-} and $S_2O_3^{2-}$ was evaluated and applied to various marine ecosystems (Table 1).

The measurements extend the number of studied environments and show that in most cases the concentrations of SO_3^{2-} and $S_2O_3^{2-}$ are in the sub-micromolar range with maximum values not exceeding a few micromoles per liter (Tab. 1). In terms of the observed concentrations the results are clearly lower and less variable compared to previous reports (see Introduction). In terms of the concentration range, the results are in good agreement with measurements by Thamdrup et al. (1994). However, they observed generally higher concentrations of SO_3^{2-} than $S_2O_3^{2-}$. In view of the high reactivity of SO_3^{2-} towards O_2, S^0 and H_2S, and in agreement with other studies (Luther III et al., 1985; Vetter et al., 1989) higher

Table 1: Summary of SO_3^{2-} and $S_2O_3^{2-}$ concentrations in different ecosystems measured with the monobromo–bimane derivatization method.

Site	Concentration (µM)		Comments
	SO_3^{2-}	$S_2O_3^{2-}$	
Sediments			
Wadden Sea mud flat	n.d.	0 - 0.5	
Black Sea	0 - 0.1	0.1 - 0.7	63 m depth, 211 µM O_2[d]
Black Sea	0 - 0.2	0.1 - 2.3	130 m depth, <5 µM O_2[d] (RTZ)[e]
Black Sea	0.2 - 0.7	1.0 - 3.6	2045 m depth, 380 µM H_2S[d]
Chile (Concepción Bay)	0.4 - 2.6	1.3 - 5.7	sulfidic sediment (> 1mM)
Chile (shelf)	0.1 - 0.5	0.2 - 2.2	H_2S in sediment <5µM, *Thioploca*
Water Columns [a]			
Mariager Fjord	1.1	5.2	
Black Sea	n.d. (<0.05)[b]	0.09	
Solar Lake	10.8[c]	350[c]	build up of $S_2O_3^{2-}$ during stratification

n.d.: not detected; a) peak concentrations; b) values in brackets indicate the detection limits; c) maximal concentrations determined in a seasonal study; d) bottom water concentrations; e) RTZ, redox transition zone.

$S_2O_3^{2-}$ than SO_3^{2-} concentrations are more realistic. Increased extracellular concentrations of SO_3^{2-} are usually not expected in the environment. The determination of SO_3^{2-} and $S_2O_3^{2-}$ in the water column of Solar Lake showed that in some environments sulfur intermediates could accumulate to high concentrations. Earlier determinations of $S_2O_3^{2-}$ by iodometric titration (Jørgensen et al., 1979) were confirmed by the bimane method.

Sulfide Oxidation in Selected Environments

Mariager Fjord (Manuscript I)

The major biogeochemical processes were studied in the sediment and water column of the stratified Mariager Fjord. It was found that under steady state conditions the vertical fluxes of reductants from the sediment, and oxidants from the mixed layer to the chemocline were balanced. During the survey, the intrusion of oxygen containing water from the Kattegat was observed, which created transient, non-steady state conditions at the chemocline.

As a result of the enhanced mixing of oxic water with sulfidic, Fe^{2+}, and Mn^{2+} containing water from below the chemocline, sulfur intermediates accumulated. The most abundant oxidation product was S^0 (17.8 µM), followed by thiosulfate (5.6 µM) and sulfite (1.2 µM), which is consistent with results from studies on the chemical oxidation of sulfide with O_2 in the presence of trace metals. The distribution of the intermediates in the water column information about their relative turnover can be derived. For instance, elemental sulfur was found far up in the oxic water column illustrating its stability towards oxygen and suggesting a low turnover. Thiosulfate was consumed much faster, and sulfite was virtually absent even at low oxygen concentrations due to the rapid chemical oxidation by O_2.

Significant contribution of chemolithotrophs to sulfide oxidation was inferred from high rates of dark $^{14}CO_2$ fixation in the chemocline and by high rates of sulfide oxidation in bottle incubation experiments. The sulfide oxidation rate was 0.266 µmol l^{-1} min^{-1}, about 15 times higher than the chemical oxidation rate under the same conditions, and one of the highest reported for stratified water columns so far.

Kinetic calculations of the chemical oxidation of sulfide, based on actual conditions in the chemocline revealed, that under steady state conditions with a narrow chemocline and low reactant concentrations, biological sulfide oxidation may account for more than 88% of the total sulfide oxidation. Under non-steady state conditions the contribution of chemical sulfide oxidation increased.

Thioploca-dominated surface sediments (Manuscripts II, III, IV)

Microbiology: The surface sediments in the upwelling area off central Chile are characterized by high sedimentary sulfate reduction rates, minimal gradients in interstitial sulfate, and low porewater sulfide concentrations. Dense mats of large, nitrate storing sulfur bacteria of the genus *Thioploca* cover the sediments in this area and potentially have a great influence on the biogeochemical sulfur and nitrogen cycles. Incubation experiments with natural *Thioploca* samples were performed to determine their physiology. The experiments showed that sulfide is efficiently oxidized to intracellular elemental sulfur, and is further oxidized to sulfate in a second, independent step it. Ammonium is the main product from the reduction of intracellular nitrate. Experiments with ^{14}C-radiolabeled acetate and HCO_3^- showed that *Thioploca* can take up both compounds for biomass formation, suggesting that *Thioploca* spp. are facultative chemolithoauto-mixotrophs.

Ecology: Ecological aspects of *Thioploca* were investigated in a small flume system where undisturbed sediment cores were incubated. Upon addition of nitrate and nitrite to the flume water *Thioploca* exhibited positive chemotaxis, and both compounds were rapidly taken up. No chemotaxis was observed towards nitrous oxide (N_2O), which is consistent with the previous observation that *Thioploca* does not reduce nitrate to dinitrogen, but to ammonium. Thus, *Thioploca* does not contribute to the large denitrification, which is usually observed in upwelling areas. In contrary, NO_3^- microsensor measurements revealed that in sediments with a high density of *Thioploca* most of the nitrate was taken up above the sediment surface. Sedimentary denitrifying organisms may thus be outcompeted for nitrate.

The concept of the vertical shuttling of *Thioploca* was confirmed by measurements of the intracellular S^0 and NO_3^- concentrations in individual filaments from different sediment depths. For the short cell morphotype *Thioploca* it could be shown that the average S^0 concentration increased with depth, whereas the average NO_3^- concentration decreased. The trend was less clear for *T. araucae* and *T. chileae*, but, here the lowest average NO_3^- and the highest S^0 concentrations were also found in the deepest samples.

Geochemistry: Only low concentrations of the dissolved sulfur intermediates thiosulfate and sulfite were measured despite high rates of sulfate reduction and efficient reoxidation of the produced sulfide, suggesting a tight coupling between the sulfur intermediate producing and consuming processes. Concentrations of thiosulfate were highest at the sediment water interface and were mostly in the sub-micromolar range. Sulfite concentrations were invariably low and did not show a trend with depth. Two different forms and distribution patterns were differentiated for elemental sulfur. Intracellular sulfur was highest at the sediment surface and decreased exponentially with depth, clearly reflecting the biomass distribution of

Thioploca. However, most of the sedimentary sulfur pool was located extracellular. The distribution of thiosulfate, elemental sulfur and dissolved iron suggested that the reaction of sulfide with iron(III)oxides may be an important pathway for sulfide oxidation and sulfur intermediate formation.

List of Publications

Contribution to the Manuscripts Presented in this Thesis

Zopfi J., Ferdelman T. G., Jørgensen B. B., Teske A., and Thamdrup B. Influence of water column dynamics on sulfide oxidation and other major biogeochemical processes in the chemocline of Mariager Fjord (Denmark). *Mar. Chem.*

Data equally provided by all contributing authors. JZ, sulfur speciation, calculations and interpretation of the data, concept and writing of the manuscript. (in press)

Otte S., Kuenen J. G., Nielsen L. P., Pearl H. W., Zopfi J., Schulz H. N., Teske A., Strotmann B., Gallardo V. A., and Jørgensen B. B. (1999) Nitrogen, carbon and sulfur metabolism in natural *Thioploca* samples. *Appl. Environ. Microbiol.* **65**, 3148-3157.

SO, JGK, incubation experiments and writing of the manuscript; ^{15}N experiments LPN; HWP. autoradiography; JZ, part of inc. experiments with SO, measurements of sulfur compounds, method development for low µM SO_4^{2-} determination at seawater Cl^- conc. HNS, AT, BS, VAG, BBJ provided helpful advise and support.

Zopfi J., Kjær T., Nielsen L. P., and Jørgensen B. B. Ecology of *Thioploca* spp.: NO_3^- and S^0 storage in relation to chemical microgradients and influence on the sedimentary nitrogen cycle. *Appl. Environ. Microbiol.*

JZ, sulfur measurements, calculations and data interpretation, concept and writing of the manuscript. TK, microsensor measurements; LPN nitrate measurements; BBJ, *Thioploca* picking and species determination. (submitted)

Zopfi J., Böttcher M. E., and Jørgensen B. B. Early diagenesis and isotopic biogeochemistry of sulfur in *Thioploca*-dominated off Chile. *Geochim. Cosmochim. Acta.*

JZ, all data besides stable isotope measurements, interpretation, concept and writing of the manuscript with editorial help of the co-authors; MEB, stable isotope measurements. (prepared for submission)

Further Publications and Manuscripts in Preparation

Zopfi J. and Jørgensen B. B. Speciation and transformation of sulfur intermediates in Black Sea sediments from the Romanian shelf to the abyssal plain. *Mar. Chem.* (in prep.)

Jørgensen B. B., Weber A., and Zopfi J. Sulfate reduction and anaerobic methane oxidation in Black Sea sediments. *Deep Sea Res.* (submitted)

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