Distribution and Diversity of Chemolithoautotrophic Microorganisms in Pelagic Redoxclines

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Sabine Glaubitz, geb. am 22.06.1978 in Ilmenau

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Gutachter

Dr. habil Matthias Labrenz

Leibniz-Institut für Ostseeforschung Warnemünde

Biologische Meereskunde

Dr. habil Hans-Peter Grossart

Leibniz-Institut für Gewässerökologie und Binnenfischerei

Abteilung Limnologie geschichteter Seen (Neuglobsow)

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Abstract

Physicochemically stratified marine water bodies are characteristic for, e. g., the Black Sea, the central Baltic Sea, or the Cariaco Trench. The stratification is due to gradually increasing salinities with depth or differences in temperature, preventing vertical mixing of the water bodies below the halocline or thermocline, respectively. This leads to oxygen consumption in deeper waters and the development of anoxic areas, which is usually also accompanied by hydrogen sulfide accumulation due to dissimilatory sulfate reduction activities. The transition zone between oxygenated and anoxic, sulfidic waters refers to as the pelagic redoxcline and features steep gradients in nutrient availability and redox conditions. Especially the oxic-anoxic interfaces are usually characterized by enhanced microbial abundances and activities. One of the characteristic features of marine pelagic redoxclines are high dark CO_2 fixation rates, often determined below the chemocline, which is defined as the shallowest appearance of sulfide.

It was early hypothesized that these activities result from chemolithoautotrophic production, but other mechanisms like anaplerotic reactions could also play a role. Microbial communities of pelagic redoxclines in anoxic basins are usually dominated by putative chemolithoautrotrophic denitrifiying *Epsilonproteobacteria*, but a correlation between maximal abundances of this potential key player and apparent CO_2 fixation maxima could never been shown. To fathom this discrepancy MICRO-CARD-FISH analyses were applied, which revealed that this phylum constituted indeed approximately 70% of the autotrophic community at the CO_2 fixation maximum in pelagic redoxclines of the central Baltic Sea and the Black Sea, respectively. Interestingly, only maximal 65% of those cells were identified as autotrophs. This raises the question of a potential microdiversity or metabolic versatility of this group and lead to the hypothesis that this phylum may fulfill more functions in the ecosystem as hitherto presumed. Though the important role of *Epsilonproteobacteria* for chemolithoautrotrophic production was confirmed for the investigated pelagic redoxclines, a detailed description of chemolithoautotrophic assemblages was still lacking.

A substantial aim of this thesis was to identify active microbial assemblages in pelagic redoxclines, covering the chemolithoautotrophic, putative mixo-, and heterotrophic potential of a natural community. For the detection of active prokaryotes, the rRNA-based stable isotope probing (RNA-SIP) method was applied. This approach is based on the incorporation of ^{13}C-labeled substrates into the 16S rRNA as a phylogenetic marker. To study the composition of the chemolithoautotrophic community [^{13}C]-bicarbonate was added to natural communities originating from the CO_2 fixation maximum of pelagic redoxclines of the central

Baltic Sea and the Black Sea. Moreover, the model substrate [^{13}C]-pyruvate was chosen to investigate the mixotrophic and heterotrophic potential of the strain *Sulfurimonas* sp. GD1 and a natural community taken from a pelagic redoxcline of the central Baltic Sea.

As main results distinct taxa of denitrifying *Epsilonproteobacteria*, related to *Sulfurimonas denitrificans* were identified as chemolithoautotroph in pelagic redoxclines of the central Baltic Sea and the Black Sea. As earlier demonstrated by MICRO-CARD-FISH analyses, the chemolithoautotrophic epsilonproteobacterial community in the Baltic Sea was exclusively constituted by the single subgroup GD17. In this thesis a similar situation for a Black Sea redoxcline was observed. The data strongly suggest that autotrophic *Epsilonproteobacteria* are mainly represented by one subcluster or even one single species. Moreover, in both systems different representatives of *Gammaproteobacteria*, belonging to the gammaproteobacterial sulfur-oxidizer cluster (GSO) were detected as actively fixing CO_2. Close relatives of these organisms were repeatedly detected in sulfide-rich marine habitats and assumed to be fulfilling a key function in sulfide removal. Especially in the Black Sea a surprisingly high diversity of this group was detected, albeit only a few representatives were identified as chemolithoautotrophs. The epsilonproteobacterial strain *Sulfurimonas* sp. GD1 was found to exhibit mixotrophic growth as earlier demonstrated. Laboratory experiments under denitrifying conditions revealed that GD1 did incorporate pyruvate into amino acids and fatty acids, but not into the RNA. The same was found for a natural community of a pelagic redoxcline in the central Baltic Sea; despite an apparent uptake of radioactive pyruvate, no labeling of nucleic acids was detectable.

This thesis provided the first detailed description of chemolithoautotrophic communities from pelagic redoxclines of two different anoxic basins. The contribution of *Gammaproteobacteria* and *Epsilonproteobacteria* to dark CO_2 fixation confirms the findings of numerous studies, in which those organisms were assigned to important biogeochemical cycles, relevant in oxygen-depleted, sulfide-rich habitats. Moreover, the hypothesized metabolic versatility of the GD17 cluster could at least partially be demonstrated, emphasizing the extensive ecological role of this key group.

Kurzfassung

Physiko-chemisch geschichtete marine Wasserkörper sind charakteristisch für beispielsweise das Schwarze Meer, die zentrale Ostsee oder das Cariaco-Becken. Die Schichtung, eine Folge unterschiedlicher Salzgehalte oder Temperaturen, die eine vertikale Durchmischung der Wasserkörper unterhalb der Halo- beziehungsweise Thermokline verhindern. Die daraus resultierende Sauerstoffzehrung im Tiefenwasser führt zur Bildung anoxischer Bereiche, die üblicherweise durch eine Akkumulation von Schwefelwasserstoff, eine Folge der dissimilatorischen Sulfatreduktionsaktivitäten, begleitet wird. Die Übergangszone zwischen sauerstoffhaltigen und anoxischen, sulfidreichen Wasserschichten wird als pelagische Redoxkline bezeichnet und ist durch steile Gradienten in Nährstoffverfügbarkeiten und Redoxbedingungen gekennzeichnet. Insbesondere die oxisch-anoxische Grenzschicht ist durch erhöhte mikrobielle Abundanzen und Aktivitäten gekennzeichnet. Eines der charakteristischen Merkmale mariner pelagischer Redoxklinen sind hohe CO_2-Dunkelfixierungsraten, die oftmals unterhalb der Chemokline gemessen werden.

Es wurde früh vermutet, dass dies ein Ergebnis chemolithoautotropher Produktion ist, jedoch können auch andere Mechanismen, wie anaplerotische Reaktionen eine Rolle spielen. Mikrobielle Gemeinschaften pelagischer Redoxklinen in anoxischen Becken werden durch vermutlich chemolithoautotrophe, denitrifizierende *Epsilonproteobacteria* dominiert, eine Korrelation zwischen der Verteilung dieser Schlüsselgruppe und CO_2-Fixierungsraten konnte jedoch bisher nie nachgewiesen werden. Um diesen Widerspruch zu erforschen, wurden MICRO-CARD-FISH-Analysen durchgeführt. Diese ergaben, dass 70% der autotrophen Gemeinschaft in der Tiefe des CO_2-Fixierungsmaximums durch dieses Phylum repräsentiert wird, wobei interessanterweise nur maximal 65% dieser Zellen als autotroph identifiziert wurden. Das wirft die Frage nach einer potentiellen Mikrodiversität beziehungsweise einer metabolischen Vielseitigkeit auf: Dieses führt zu der Hypothese, dass dieses Phylum mehr Funktionen in dem Ökosystem ausfüllen könnte als bisher angenommen. Obgleich die wichtige Rolle der *Epsilonproteobacteria* in der chemolithoautotrophen Produktion in den untersuchten pelagischen Redoxklinen bestätigt wurde, mangelte es bisher immer noch an einer detaillierten Beschreibung der chemolithoautotrophen Gemeinschaft.

Das Ziel dieser Dissertation bestand daher in der Identifizierung aktiver mikrobieller Gemeinschaften, die das chemolithoautotrophe und mutmaßlich mixo- und heterotrophe Potential einer natürlichen Gesellschaft abdecken. Dafür wurde die rRNA-basierte Stabile-Isotopen-Technik (SIP) angewendet, die auf der Aufnahme eines ^{13}C-markierten Substrates in den phylogenetischen Marker 16S rRNA beruht. Für die Untersuchung der

chemolithoautotrophen Gemeinschaft wurden Wasserproben, die aus dem CO_2-Fixierungsmaximum pelagischer Redoxklinen der Ostsee und des Schwarzen Meeres stammten, mit [^{13}C]-Bikarbonat inkubiert. Des Weiteren wurde das Modellsubstrat [^{13}C]-Pyruvat ausgewählt, um das mixo- und heterotrophen Potentials des Stammes *Sulfurimonas* sp. GD1 und einer natürlichen Gemeinschaft aus einer pelagischen Redoxkline der Ostsee zu untersuchen.

Als Hauptergebnisse dieser Arbeit wurden unterschiedliche Taxa der denitrifizierenden *Epsilonproteobacteria*, verwandt mit *Sulfurimonas denitrificans,* als chemolithoautotroph in pelagischen Redoxklinen der Ostsee und des Schwarzen Meeres identifiziert. Wie bereits durch MICRO-CARD-FISH-Analysen gezeigt, bestand die chemolithoautotrophe, epsilonproteobakterielle Gemeinschaft in der Ostsee ausschließlich aus der einzelnen Untergruppe GD17. In der vorliegenden Dissertation wurde auch eine vergleichbare Situation in einer Redoxkline des Schwarzen Meeres beobachtet, was darauf hinweist, dass die *Epsilonproteobacteria* ebenfalls durch eine einzelne Gruppe oder sogar eine einzelne Art repräsentiert werden. Darüber hinaus wurden in beiden Systemen Vertreter der *Gammaproteobacteria*, die zu den gammaproteobakteriellen Schwefeloxidierern (GSO) gehören, als CO_2-Fixierer identifiziert. Nahe Verwandte dieser Organismen wurden mehrfach in sulfidischen Standorten nachgewiesen und erfüllen dort mutmaßlich eine Schlüsselrolle im Schwefelkreislauf. Insbesondere im Schwarzen Meer wurde eine überraschend hohe Diversität innerhalb dieser Gruppe detektiert, obschon nur wenige Vertreter dieser als chemolithoautotroph identifiziert wurden. Für den Stamm *Sulfurimonas* sp. GD1 wurde nachgewiesen, dass Pyruvat in Amino- und Fettsäuren, jedoch nicht in Nukleinsäuren aufgenommen wurde. Ähnliches wurde auch für eine natürliche mikrobielle Gemeinschaft einer pelagischen Redoxkline der zentralen Ostsee gezeigt. Trotz einer messbaren Aufnahme des radioaktiven Pyruvats konnte keine Markierung der Nukleinsäuren nachgewiesen werden.

In dieser Arbeit erfolgte die erste detaillierte Beschreibung chemolithoautotropher Gemeinschaften pelagischer Redoxklinen zweier anoxischer Becken. Die Beteiligung der *Gammaproteobacteria* und der *Epsilonproteobacteria* an der CO_2-Dunkelfixierung bestätigen frühere Ergebnisse, in denen diesen Organismen eine tragende Rolle in wichtigen biogeochemischen Zyklen, die relevant in sauerstoffarmen, sulfidreichen Habitaten sind, zugewiesen wurde. Zudem wurde die vermutete metabolische Vielseitigkeit der GD17-Gruppe zumindest teilweise bewiesen, was die beträchtliche ökologische Rolle dieser Schlüsselgruppe unterstreicht.

Introduction

The hydrography of the Baltic and the Black Sea

The Baltic Sea, our area of investigation, is among the largest brackish basins worldwide with periodically occurring anoxic conditions in the Baltic proper (Figure 1). This inland sea covers an area of 412,560 km^2 and is surrounded by a number of highly developed industrial nations. The drainage area of the Baltic Sea, which is about 1,700,000 km^2 large, has a population of approximately 85 million people. The depth averages 52 m and its mean volume accounts for 21,631 km^3 (HELCOM, 2003, 2009).

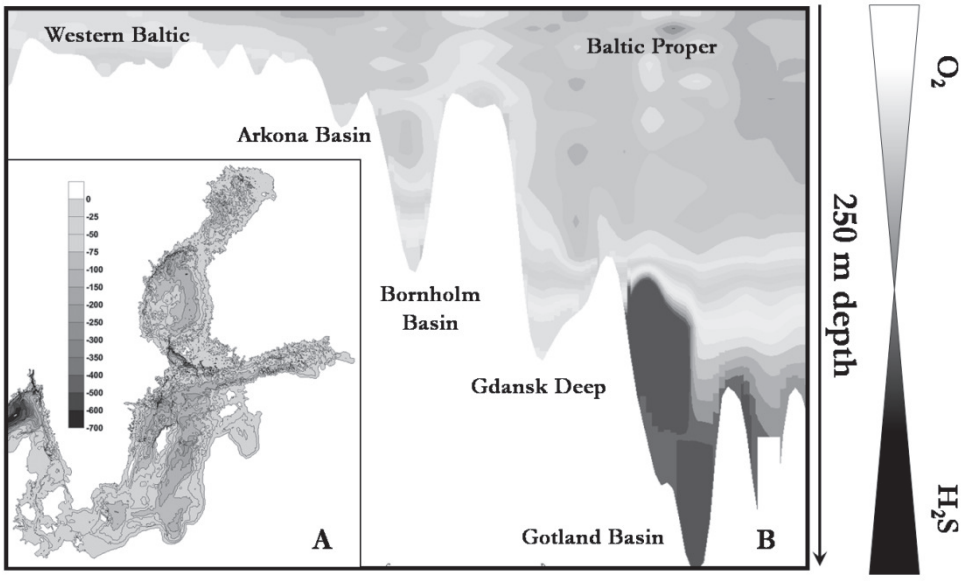

Figure 1 Oxic-anoxic regimes in the central Baltic Sea.

(A) Bathymetric map of the Baltic Sea. (B) Bottom structure of the Baltic proper. Oxygen and hydrogen sulfide concentrations are indicated by grey shading. Modified from Stockholm University.

The basin of the Baltic Sea is featured by a characteristic bottom structure with alternating sills and deeps (Figure 1B). The largest Basin is the Gotland Deep (248 m depth) and the deepest the Landsort Deep (459 m depth). By formation of a stable halocline in the Baltic proper, the vertical ventilation of water masses in these basins is impaired. The exchange of marine water masses is restricted to the Kattegat, which is the exclusive connection to the North Sea. Usually inflow events from North Sea water can cause a complete ventilation of the Baltic water masses (Rheinheimer, 1995; Meier et al., 2006), but the frequencies of those events changed within the last decades. Since the 1970' just a few

complete exchanges of Baltic water could be recorded (Matthäus and Franck, 1992). In inter-inflow periods, so-called stagnation periods, the input of freshwater by precipitation and river output is considerably higher than the inflow of saltwater from marine system. This imbalance leads to a permanent stratification of the water column by the development of a stable halocline, usually located at a depth of 70 m (Matthäus and Franck, 1992; Samuelsson, 1996). In postindustrial times the Baltic Sea is strongly influenced by a pronounced eutrophication, caused by riverine output of sewage and industrial waste (Fonselius and Valderrama, 2003; HELCOM, 2003, 2009), promoting growth of phytoplankton, and thus the primary production (Rheinheimer, 1998). The remineralization of the produced organic matter in sediments is an oxygen-consuming process, and hence directly influencing the establishment of anoxic bottom waters (Rheinheimer, 1998) and the accumulation of sulfide.

The Black Sea, the largest permanently anoxic system worldwide, is a semi-enclosed inland sea, characterized by one deep basin and a pronounced shelf-region in the north-west (Figure 2). It covers an area of 423,000 km^2 and the maximal depth accounts for 2243 m (Murray et al., 2005). The inflow of oxygenated saltwater, originating from the Mediterranean, is restricted to the shallow (maximal 93 m) and narrow (0.76 - 3.60 km) Bosporus strait.

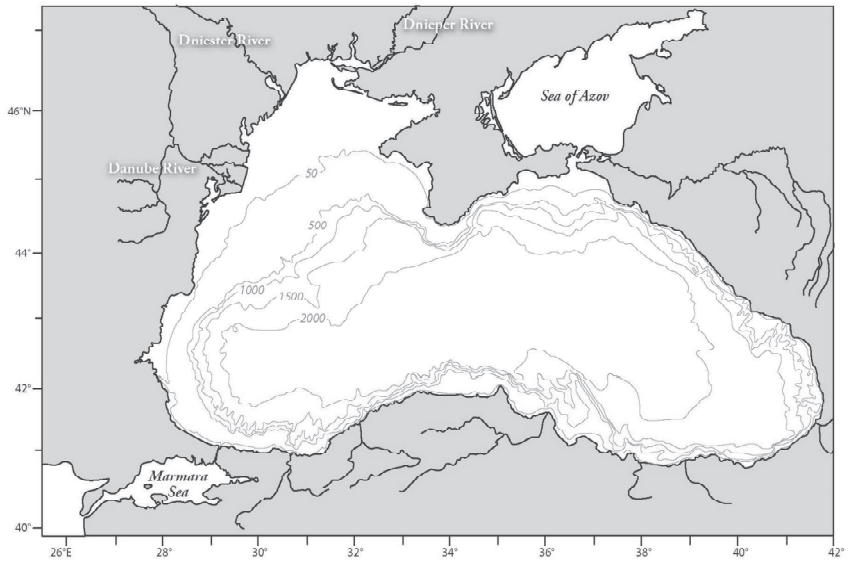

Figure 2 Bathymetric map of the Black Sea.
Modified from Wakeham et al. (2007)

Like in the Baltic Sea, the Black Sea is strongly influenced by freshwater inflow from numerous, highly polluted river systems and precipitation, leading to a surface layer strongly reduced in salinity. In general, the input of freshwater is supposed to be twice as much as the

inflow of saltwater from the Bosporus. In consequence, a stable pycnocline is formed, resulting in a permanent stratification of the water column (Murray and Yakushev, 2006). The stratification and the negative water balance is leading to a distinct zonation of the water column; like in the Baltic Sea the surface waters are well-oxygenated, the deeper waters are oxygen-deficient and sulfidic. The Black Sea features a relatively thick water layer (approximately 50m), where oxygen and sulfide concentrations are extremely low (Murray et al., 2005). About 85 % of the water masses are thought to be permanently anoxic (Yakushev et al., 2008). Contrary to the situation in the Baltic Sea, those conditions are supposed to persist within geological periods; the age of the sulfidic zone was estimated to persist approximately 7500-7800 years (Jones and Gagnon, 1994; Arthur and Dean, 1998). In recent years, the Black Sea is recognized as a contemporary analogue to past paleooceans and got into the focus of several disciplines of oceanographic sciences (Degens and Stoffers, 1976).

Pelagic redoxclines in stratified aquatic systems

Oxygen, the primary terminal electron acceptor for respiratory processes of most organisms is supposed to be the key parameter for biogeochemical cycles, involved in carbon and nitrogen transformations (Karstensen et al., 2008). Because of the high redox potential between $NADH+H^+$ and O_2 the aerobic transformations of organic substrates yield high amounts of energy (Brune et al., 2000). In highly productive aquatic habitats, i.e. upwelling systems, where oversupplies of nutrients and organic matter from enhanced primary production exists, oxygen can be consumed by aerobic heterotrophic processes up to extinction, resulting in oxygen deficient systems. The occurrence of these oxygen-minimum zones (OMZ) is favored by oxygen-consuming biogeochemical processes, but also by physical parameters like decreased replenishment of oxygenated water due to weak circulation processes (Helly and Levin, 2004; Karstensen et al., 2008).

Typical OMZ are found in the Arabian Sea (Devol et al., 2006), in the tropical Atlantic ocean as well as in the Pacific ocean (Karstensen et al., 2008; Stramma et al., 2008; Lam et al., 2009). In general the OMZ are found in intermediate or shallow waters, usually at highly productive areas with usually increasing oxygen concentrations in the bathypelagial (Karstensen et al., 2008). The absence of oxygen can favor the accumulation of sulfide produced by sulfate reducing activities in sediments (Tuttle and Jannasch, 1973a; Gocke, 1989; Piker et al., 1998). The occurrence of this toxic compound in shelf waters is a well known phenomenon, and it is known to cause mass extinction of metazoans (Copenhagen, 1953; Gray et al., 2002; Lavik et al., 2009). However, this potential electron donor can be a potent source of energy for a number of prokaryotes, fueling chemolithoautotrophic processes in

marine habitats (Ruby et al., 1981; Nelson and Jannasch, 1983; Jannasch et al., 1991; Mandernack and Tebo, 1999; Wirsen et al., 2002). Recently it was reported that sulfur-oxidizing prokaryotes, phylogenetically belonging to the *Gammaproteobacteria* and *Epsilonproteobacteria* can form pronounced blooms in presence of sulfide in OMZ. These highly abundant sulfur-oxidizing organisms were capable of removing significant amounts of the toxic sulfide and establishing a sulfide-free, anoxic buffer zone (Lavik et al., 2009).

The focus of this work were oxic-anoxic transition zones in stratified aquatic habitats. This special habitat, referred to as pelagic redoxclines, is characterized by alternating redox conditions throughout depth (Hallberg, 1972) and were reported for numerous pelagic water columns as in the Black Sea (Sorokin, 1964; Jørgensen et al., 1991), the Cariaco Basin (Tuttle and Jannasch, 1973b, 1979; Taylor et al., 2001), river salt wedges (Casamayor et al., 2001), the Mariager Fjord (Zopfi et al., 2001), or stratified freshwater or saline lakes (Jørgensen et al., 1979; Casamayor et al., 2008). Oxic-anoxic interfaces are characteristic for a variety of habitats, e.g. flooded soils (Laanbroek, 1990), sediments (Sass et al., 1996, 1997), and even intestinal tracts of animals (Horn et al., 2003). The stratification, a consequence of limited vertical mixing of the water column, is due to different hydrographic characteristics depending on the respective aquatic system (Fanning and Pilson, 1972; Holmen and Rooth, 1990; Matthäus and Franck, 1992; Kruse and Rasmussen, 1995; Özsoy and Ünlüata, 1997; Yakushev et al., 2008).

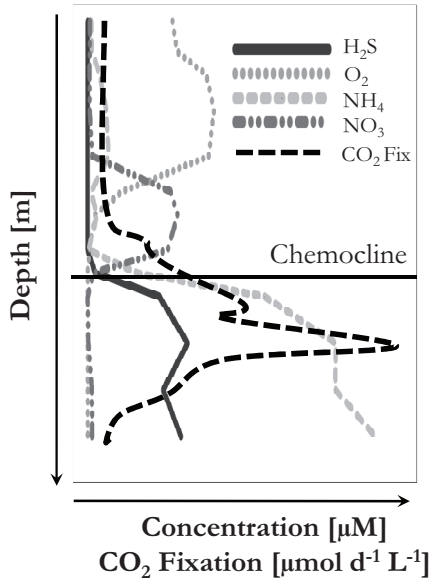

Figure 3 Schematic illustration of a characteristic depth profile of a redoxcline in the central Baltic Sea. Concentrations of oxygen, sulfide, nitrate, and ammonia as well as CO2 fixation rates are provided. The line marks the chemocline.

Major factors which are influencing a stable stratification are differences in density, leading to pycnoclines, differences in temperature, leading to thermoclines, and differences in salinity, leading to haloclines. Redoxclines are characterized by steep gradients in physicochemical parameters and the availability of inorganic nutrients, with change from oxidizing to reducing conditions (Lepland and Stevens, 1998; Neretin et al., 2003). Oxygen, as the primary terminal electron donor decreases to zero and sulfide accumulates below the chemocline (Rheinheimer et al., 1989), which is defined as the zone of first sulfide appearance (Figure 3).

Microbial processes in stratified aquatic systems

Interfaces and transition zones are usually areas of enhanced microbial abundances and activities, which is caused by steep gradients of physicochemical parameters, fueling energy-yielding processes. In aquatic systems the stratification of the water column is reflected by a pronounced stratification of the microbial community, including a shift in physiological capacities (Meyer-Reil, 2005). The energy-yielding and element-transforming pathways alternate within the transition zone and are presumably coupled to each other (Figure 4).

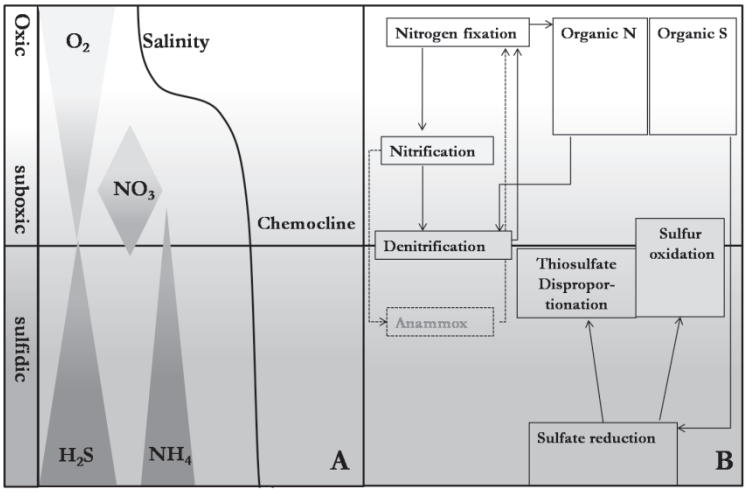

Figure 4 Element transformations at pelagic redoxclines.

(A) Typical physic-chemical conditions in pelagic redoxclines. (B) Simplified scheme of microbial transformation processes in the water column.

These processes at pelagic redoxclines have a significant impact on local as well as presumably global biogeochemical cycles. In the following paragraphs the nitrogen and sulfur transforming processes will be described in detail.

Nitrogen cycles

Bioavailable nitrogen compounds are supposed to be the major limiting factors in marine systems (Flynn and Butler, 1986; Pitkanen and Tamminen, 1995; Granéli and Granéli, 2008). In coastal shelf regions or in estuarine systems like the Baltic Sea the concentrations of nitrogen species is mostly determined by riverine input, atmospheric deposition and nitrogen fixation (Voss et al., 2005).

A typical depth profile of a pelagic redoxcline is characterized by an alternating distribution of nitrogen species (Rheinheimer et al., 1989; Granéli and Granéli, 2008). In the suboxic zone nitrate peaks sharply, and ammonia concentrations are usually low. Below the chemocline the nitrate concentrations are below the detection limit and ammonia increase steadily with depth (Rönner and Sörensson, 1985; Rheinheimer et al., 1989; Brettar and Rheinheimer, 1991) (Figures 3, 4A). This distribution reflects the alteration from aerobic ammonia oxidation (nitrification) above the chemocline and the anaerobic nitrate respiration (denitrification) below the chemocline (Figure 4B).

The ammonia in the redoxcline originates from the remineralization of organic matter. The initial step of the nitrification is mainly catalyzed by *Beta- and Gammaproteobacteria* (McCaig et al., 1994; Voytek and Ward, 1995; Kowalchuk and Stephen, 2001) or *Crenarcheota* from the Marine Cluster I (Francis et al., 2005; Wuchter et al., 2006). Though ammonia-oxidizing bacteria (AOB) were detected in pelagic redoxclines of the central Baltic Sea, the overall abundance is rather low, with maximal cell counts of 3.9% of total Prokaryotes (Bauer, 2003). The crenarchaeal abundances are considerably higher, and hence, this potential key group is presumably dominating the ammonia oxidizing community in Baltic Sea pelagic redoxclines (Labrenz et al., 2010). Recently a crenarcheon was isolated and described as a mesophilic, ammonia-oxidizing autotroph (Könneke et al., 2005). Relatives of this *Candidatus* 'Nitrosopumilus maritimus' SCM1 are supposed to be ubiquitous distributed worldwide, potentially playing a key role in global nitrogen budget (Delong, 1992; Coolen et al., 2007; Agogué et al., 2008; Erguder et al., 2009). In the suboxic zone of Baltic Sea redoxcline a distinct cluster, clearly separated from *Candidatus* 'Nitrosopumilus maritimus', but within the marine group I was recently discovered and supposed to be a key player in aerobic ammonia oxidation (Labrenz et al., 2007, 2010).

In the anoxic zone, where no sulfide and no oxygen are present, a second recycling process of ammonia, coupled to chemoautotrophy is possible. Via the so-called anaerobic ammonium oxidation process (anammox), exclusively driven by *Planctomycetes*, nitrite and ammonia are anaerobically converted to molecular nitrogen (Mulder et al., 1995; Van de Graaf

et al., 1995; Strous et al., 1999). This process, originally discovered from wastewater treatment plants, is present in numerous natural habitats like oceanic OMZ, sediments, anoxic basins as well as anoxic fjords, and is considered to be a significant N-loss process (Kuypers et al., 2003; Schouten et al., 2004; Thamdrup et al., 2006; Coolen et al., 2007; Lam et al., 2007; Stevens and Ulloa, 2008; Woebken et al., 2008; Lam et al., 2009). This process seems to be less important in Baltic Sea redoxclines, but after an inflow event in 2003 in the Baltic proper anammox activities could be detected (Hannig et al., 2007). In general this process is below the detection limit.

Below the chemocline the major N-loss process is the denitrification, in the Baltic Sea coupled to chemolithoautotrophy and oxidation of reduced sulfur compounds (Brettar and Rheinheimer, 1991; Jensen et al., 2009). Heterotrophic denitrification, usually proceeding in anoxic sediments, was detected in Baltic Sea redoxclines via culture-dependent methods (Brettar and Höfle, 1993; Brettar et al., 2001), but culture-independent approaches revealed that denitrification is mostly coupled to autotrophic sulfur-oxidation in this habitat (Brettar and Rheinheimer, 1991; Falk et al., 2007; Hannig et al., 2007). Nitrate is reduced to molecular nitrogen and different oxidized nitrogen species and hence, it serves as a terminal electron acceptor for anaerobic respiration processes, fueling autotrophic processes. Recently, a key player for autotrophic denitrification was identified in pelagic redoxclines, which belongs phylogenetically to *Epsilonproteobacteria* (Brettar et al., 2006). This organism, closely related to *Sulfurimonas denitrificans* and highly abundant at oxic-anoxic interfaces contributes to a major part to chemolithoautotrophic production in pelagic redoxclines of the central Baltic Sea (Grote et al., 2007, 2008).

Sulfur cycles

In oxygen-depleted systems like pelagic redoxclines or sediments the sulfur cycle is of major importance, linking aerobic and anaerobic processes as well as other element cycling processes (reviewed in Sievert et al., 2007). Sulfur may occur in numerous oxidation states; hence, these compounds presumably serve as electron donor and acceptor for a variety of microorganisms (Figure 4B).

Sulfate, the most oxidized and most stable form of sulfur, serves as a terminal electron acceptor in anoxic sediments or deeper water layers for sulfate-reducing bacteria (Jørgensen and Fenchel, 1974; Canfield, 1991; Sorokin et al., 1995), mediating one of the most important process in remineralization of organic matter (Jørgensen, 1977). The activity of the dissimilatory sulfate reductase is the major source of marine sulfide (Jørgensen and Postgate, 1982), which accumulates in the water column. Sulfate-reducing prokaryotes are supposed to

be phylogenetically divers, but most identified representatives in mesophilic systems belong to the phylum *Deltaproteobacteria* (Devereux et al., 1989; Ramsing et al., 1993; Teske et al., 1996). Hydrogen sulfide, the most reduced form of sulfur serves as an important electron donor for numerous prokaryotes (Jannasch et al., 1991; Jørgensen et al., 1991; Cardoso et al., 2006; Preisler et al., 2007). The sulfide oxidation is mostly coupled to autotrophy, and as electron acceptor usually molecular oxygen or nitrate is used. At the oxic-anoxic interface the spontaneous oxidation of H_2S to thiosulfate and elemental sulfur is proposed (Jørgensen and Bak, 1991; Sorokin, 2005). Especially thiosulfate got into the focus as a major energy-rich nutrient in pelagic redoxclines, as it serves as the primary electron donor for numerous autotrophic prokaryotes (Tuttle and Jannasch, 1973b; Ruby et al., 1981). In recent years the knowledge on the disproportionation of thiosulfate and elementary sulfur accumulated (Bak and Cypionka, 1987; Vairavamurthy et al., 1993; Finster et al., 1998; Finster, 2008; Grote, 2009), these compounds may serve as electron acceptor and donor simultaneously.

Chemolithoautotrophy

CO_2 fixation, chemo- or photosynthetically, is the basis for all life forms, and is considered to be the most important biosynthetic process on earth (Hügler et al., 2003). The process of dark CO_2 fixation refers to as chemolithoautotrophy, the energy for assimilation of inorganic carbon originates from inorganic respiratory processes. As electron donors mostly serve reduced inorganic compounds, like molecular hydrogen, reduced sulfur (e.g. H_2S, S^0, $S_2O_3^{2-}$), reduced nitrogen (NH_4^+, NO_2^-), carbon (CO, CH_4) or even metals (Fe^{2+}, Mn^{2+}) (Schlegel, 1960; Shively et al., 1998). The primary terminal electron acceptors are mostly nitrate or molecular oxygen, but also oxidized metals or oxidized sulfur-species (Kelly and Wood, 2000). The chemolithoautotrophy is a solely prokaryotic feature, which can be found in nearly all phyla of *Archaea* and *Bacteria* and is considered to be an ancient or even the earliest lifestyle. In addition to the role of chemolithoautotrophs in the global carbon cycle, these organisms play a key role in biogeochemical processes, like the global sulfur and nitrogen cycle. Chemolithoautotrophic production was reported in a number of different habitats, such as soils (Selesi et al., 2005), aquifers (Kellermann, 2008), marine and limnic systems (Ward et al., 1989), hydrothermal vents (Karl et al., 1980; Fisher et al., 1989; Mandernack and Tebo, 1999; Campbell et al., 2003), cold seeps (Duperron et al., 2007), sediments and subsurface environments (Canfield, 1991; Stevens, 1997; Thomsen and Kristensen, 1997), sulfidic caves (Engel et al., 2004; Chen et al., 2009; Dattagupta et al., 2009) and pelagic redoxclines in anoxic basins and fjords (Sorokin, 1964; Tuttle and Jannasch, 1979; Juniper and Brinkhurst, 1986; Gocke, 1989; Jannasch et al., 1991; Taylor et al., 2001; Zopfi et al., 2001).

During earth's history different pathways for fixation of inorganic carbon dioxide evolved. The most common, the Calvin Cycle is employed by all higher plants, cyanobacteria and a number of chemolithoautotrophs. The key enzyme, the Ribulose-Bisphosphate-Carboxylase/Oxygenase (RuBisCO) is supposed to be the most abundant protein on Earth. However, the Calvin Cycle is an energy-demanding process, for each triosephosphate approximately 9 ATP and 6 $NADPH+H^+$ are required (reviewed in (Shively et al., 1998)). In energy-depleted systems like pelagic redoxclines, alternative pathways as the reductive tricarboxylic acid (rTCA) (Evans et al., 1966) or the 3-hydroxypropionate pathway (Holo, 1989) may play a more significant role.

The rTCA, the reverse running citrate-cycle, was originally identified in *Chlorobium limicola* (Evans et al., 1966; Fuchs et al., 1980a; Summons and Powell, 1986), but the presence of this ancient process was suggested for several thermophilic as well as sulfate-reducing prokaryotes (Zhang et al., 1996; Hügler et al., 2003; Williams et al., 2006). In the last decade the knowledge about the significance of this cycle in aquatic habitats accumulated; clone libraries, enzyme activity approaches, analyses of stable carbon isotope signatures as well as genome analyses revealed that this pathway is the prevalent pathway in chemoautotrophic *Epsilonproteobacteria* (Campbell et al., 2003; Campbell and Cary, 2004; Hügler et al., 2005; Takai et al., 2005; Sievert et al., 2008; Grote, 2009). The rTCA cycle is much more energy-efficient, for each synthesized triosephosphate just 12 reduction equivalents and 5 ATP is needed (Evans et al., 1966; Buchanan and Arnon, 1990; Campbell and Cary, 2004).

For pelagic redoxclines it is considered that chemolithoautotrophic production is presumably coupled to denitrification and sulfur oxidizing processes (Brettar and Rheinheimer, 1991; Labrenz et al., 2005). Since at depths where the maximal dark CO_2 fixation rates occur nitrate concentrations are below the detection limit (Figure 3), it is still in discussion whether alternative pathways or electron acceptors could be employed as driving force for chemolithoautotrophy. The inorganic fermentation, the disproportionation of thiosulfate or sulfur could be a possible explanation (Jost et al., 2010), but also the reduction of oxidized metals could be an important driver of chemolithoautotrophic production.

Despite the fact that chemolithoautotrophs are able to grow with inorganic carbon as the sole carbon source, the incorporation of organic low-molecular compounds into their biomass to a certain extent is possible (Taylor et al., 1971; Matin, 1978; Jannasch et al., 1991). Even in obligate chemolithoautotrophs, which are reliant on autotrophy, up to 10% of the total cell carbon can originate from additional organic carbon sources (Matin, 1978; Jannasch et al., 1991). Recently enhanced denitrification rates and sulfate formation rates were reported

after addition of acetate to an enrichment culture of obligate chemolithoautotrophs under denitrifiying and sulfur-oxiding conditions (Cardoso et al., 2006). The addition of low-molecular organic acids may even increase cell-yield of chemolithoautotrophs under CO_2 limiting conditions, the amount of incorporated substrate correlated directly with the increase in growth (Kuenen and Veldkamp, 1973).

Chemolithoautotrophic assemblages in pelagic redoxclines

The highest dark CO_2 fixation rates in anoxic basins are usually detected directly below the chemocline. The rates may account for up to 30% in the Baltic Sea (Detmer et al., 1993), and in the Cariaco Basin 10 % or occasionally up to 333% (Taylor et al., 2001). Numerous studies investigating the composition of prokaryotic assemblages in pelagic redoxclines but also other mesophilic, sulfidic habitats provided evidence for abundance and prevalence of putative chemoautotrophic *Epsilon-* and *Gammaproteobacteria*. In the following, these two potential key groups will be presented in more detail.

Epsilonproteobacteria

The *Epsilonproteobacteria*, a phylum within the *Proteobacteria*, were originally discovered as human pathogens. The most common representatives and thus, well described, are *Helicobacter pylori* (Tomb et al., 1997; Marais et al., 1999), which is the major cause for gastritis and *Campylobacter jejuni*, causing gastroenteritis and food-poisoning (Blaser, 1997; Parkhill et al., 2000).

In recent years the knowledge of this phylum in extreme, sulfide-rich habitats accumulated. An increasing number of 16S rRNA sequences of uncultured *Epsilonproteobacteria* were found in a variety of terrestic and aquatic habitats worldwide, as hydrothermal vents (Polz and Cavanaugh, 1995; Corre et al., 2001; Nakagawa et al., 2005a, 2005b), cold seeps (Li et al., 1999; Inagaki et al., 2002; Heijs et al., 2005), sulfidic spring caves (Engel et al., 2003, 2004), oil reservoirs (Grabowski et al., 2005; Sette et al., 2007), but also in anoxic marine areas, pointing to a prevalence of this phylum in systems with high dynamic changes in physicochemical conditions. Besides free-living representatives also symbionts with metazoan hosts are known (Campbell et al., 2003; Suzuki et al., 2005; Nakagawa and Takai, 2008). In several studies a pronounced metabolic versatility of members within the *Epsilonproteobacteria* was hypothesized, including heterotrophic and chemolithoautotrophic lifestyles, the capability of reducing nitrate, nitrite and sulfate, as well as the utilization of reduced sulfur species, molecular hydrogen and formate as electron donor.

The prevalence and importance of the *Epsilonproteobacteria* in mesophilic sulfidic habitats was proven in a couple of studies in the last years. Representatives, mainly closely related to *Sulfurimonas denitrificans* were found to be abundant in pelagic redoxclines of the Black Sea (Vetriani et al., 2003; Lin et al., 2006), the Baltic Sea (Labrenz et al., 2004, 2005; Grote et al., 2007; Labrenz et al., 2007), the Cariaco Basin (Madrid et al., 2001; Lin et al., 2006), but also in oxygen-minimum zones like the Namibian shelf (Lavik et al., 2009) or the hypersaline Urania basin in the Mediterranean Sea (Borin et al., 2009). In quantitative terms the *Epsilonproteobacteria* can reach abundances in sulfidic waters up to 27% in the Cariaco Basin and 20% in the Black Sea (Lin et al., 2006). Similar abundances of these organisms were determined by Grote et al. (2008); the authors determined maximal epsilonproteobacterial abundances of 27% in the Baltic and 35 % in the Black Sea. By application of a specific oligonucleotide probe the authors stated that at least in the Baltic Sea the *Epsilonproteobacteria* are mainly representated by one single cluster GD17 closely related to *Sulfurimonas denitrificans* (Grote et al., 2007), which is supposed to be a key player in autotrophic denitrification (Brettar et al., 2006). The application of a MICRO-CARD-FISH (catalyzed reporter deposition fluorescence in situ hybridization combined with microautoradiography) protocol revealed that *Epsilonproteobacteria* may contribute to a major part to the total chemolithoautotrophic production in the Black and the Baltic Sea. In the Baltic up to 77% of chemolithoautotrophs were identified as *Epsilonproteobacteria*, which were exclusively constituted of the subcluster GD17 (Grote et al., 2008). In a Black Sea redoxcline a comparable contribution of *Epsilonproteobacteria* was recognized, but more detailed information on a putative subcluster, similar to the Baltic GD17 are not available up to date. Studies of the representative Epsilonproteobacterium GD1 isolated from a central Baltic Sea redoxcline revealed that this member of the GD17 group possesses all genes for denitrification, and CO_2 fixation by the rTCA cycle, affirming the role of this organism as an autotrophic denitrifiying bacterium. Moreover, supporting autecological studies revealed that GD1 showed indeed a pronounced metabolic versatility and a chemotactical behavior towards nitrate (Grote, 2009).

Gammaproteobacteria

The subclade *Gammaproteobacteria* is the largest taxonomical group within the clade *Proteobacteria*, which are morphologically and physiologically very divers. It comprises numerous pathogens which are of major importance in public health, like representatives of the *Enterobacteriaceae* as *Escherichia coli, Salmonella enteritidis*, *Yersinia pestis*, or *Vibrio cholerae* (reviewed in Stephen, 2001).

A group of major importance is the order *Thiotrichales* with a number of obligate aerobic, chemolithoautotrophic, sulfide-oxidizing representatives like *Begiattoa* sp., *Thiotrix* sp. and *Thiomargarita namibiensis* (reviewed in Garrity et al., 2005). These organisms may form huge mats or filaments and are considered to participate to a major part in sulfur-cycling.

A second cluster, which got in the focus of numerous scientists, are unclassified *Gammaproteobacteria*, forming a phylogenetic distinct group apart the *Thiotrichales*. The so-called gamma-sulfur-oxidizer (GSO) were originally discovered as typical endosymbionts of metazoans in benthic or abyssal communities of hydrothermal vents (Felbeck, 1981; Jones, 1981), cold seeps (Heijs et al., 2005), and whale falls but also coastal sediments (Stewart et al., 2005). In recent years within this cluster numerous, putative free-living representatives were identified (reviewed in Nakagawa and Takai, 2008). In recent years the existence of this group was also proven for numerous mesophilic anoxic habitats, like the Black Sea (Vetriani et al., 2003), the Baltic Sea (Labrenz et al., 2007), the periodically anoxic fjord Saanich Inlet (Walsh et al., 2009), or marine oxygen minimum zones (Stevens and Ulloa, 2008; Lavik et al., 2009). At the Namibian upwelling system maximal abundances of 11 % of total cell counts were determined by application of a newly designed GSO-specific gene probe (Lavik et al., 2009). The coincidence of GSO cells with the occurrence of hydrogen sulfide and colloidal sulfur leaded to the hypothesis that these organisms are potentially involved in the complete oxidation of this toxic compound.

In general, cell abundances of *Gammaproteobacteria* are supposed to be rather low. Lin et al. (2006) determined maximal cell counts of 6% in the Cariaco Basin and in the Black Sea, but the specificity of the employed gene probe GAM42a (Manz et al., 1992), targeting the 23S rRNA, has been questioned in several studies (Yeates et al., 2003; Siyambalapitiya and Blackall, 2005; Grote, 2009).

However, hitherto only indirect evidences of chemolithoautotrophic growth exist; incorpotation studies (Karl et al., 1980; Fisher et al., 1989), isotopic composition of host tissues (Scott et al., 1999), enzyme activities studies (Felbeck, 1981) and metagenome (Robidart et al., 2008) and proteome (Markert et al., 2007) analyses lead to the hypothesis that these bacteria grow chemolithoautotrophically, by this way supporting their hosts with newly synthesized organic carbon (Jones, 1981; Nakagawa and Takai, 2008;). The assimilation of inorganic carbon is most probably catalyzed via the Calvin Cycle, but from proteome and metagenome analyses the additional employment of the rTCA cycle in *Candidatus* 'Endoriftia persephone' is proposed (Markert et al., 2007; Robidart et al., 2008).

Further organisms

Numerous studies suggest the existence of other putative chemolithoautotrophs in mesophilic pelagic redoxclines, which also may contribute to a distinct part to the total autotrophic production. Below the most probable taxonomical groups will be described.

In suboxic marine environments, with considerable dark CO_2 fixation rates, the contribution of ammonia-oxidizing *Crenarcheota* to chemolithoautotrophic production is imaginable. For several habitats the existence and ammonia-oxidizing activity of the *Candidatus* "Nitrosopumilus maritimus" and its relatives, respectively, was proven, leading to a revised view on nitrogen and carbon cycle in natural habitats (Wuchter et al., 2003; Könneke et al., 2005; Wuchter et al., 2006; Coolen et al., 2007; Labrenz et al., 2007; Lam et al., 2007; Labrenz, 2010). Analyses of natural radiocarbon signature provided evidence for chemolithoautotrophic growth of these cells (Ingalls et al., 2006), and [^{13}C]-bicarbonate uptake into specific archaeal lipids was demonstrated already in 2003 for a natural water sample originating from the North Sea (Wuchter et al., 2003). Genome analyses of the isolated type strain *Candidatus* "Nitrosopumilus maritimus" SCM1 (Walker et al., 2010) and fosmid libraries from *Cenarchaeum symbiosum* (Hallam et al., 2006) suggested an employment of a modified 3-hydroxypropionate cycle for fixation of inorganic carbon, providing further evidence for autotrophic growth.

In sediments as well as anoxic waters sulfate-reducing bacteria (SRB), mostly belonging to the phylum *Deltaproteobacteria* are supposed to be abundant and key organisms in carbon remineralization. Recently, the contribution of autotrophic sulfate reducing bacteria (SRB) to chemolithoautotrophic production in Black Sea anoxic waters was suggested (Neretin et al., 2007), but no direct evidence for this exists hitherto. In the Black Sea the maximum of sulfate reduction rates occurs typically below the CO_2 fixation maximum (Pimenov and Neretin, 2006). In several studies the maximal abundance of sulfate reducing organisms was determined via FISH (Durisch-Kaiser et al., 2005) or qPCR targeting the functional gene *dsrA* (Neretin et al., 2007), the maximum abundance of these organisms contributed from 3 to 7% and 1-5%, respectively, to the total cell abundance. The autotrophic potential of isolated strains was already proven (Butlin and Adams, 1947; Brysch et al., 1987; Schauder et al., 1987); as putative CO_2 fixation pathways the Acetyl-CoA-pathway as well as the reductive tricarboxylic acid pathway is suggested (Schauder et al., 1987, 1988; Londry and Des Marais, 2003; Strittmatter et al., 2009).

Shallow redoxclines in anoxic fjords or in meromictic lakes are dominated by potentially photoautotrophic sulfide-oxidizing prokaryotes, like green and purple sulfur

bacteria (Jørgensen and Postgate, 1982; Øvreås et al., 1997; Tonolla et al., 1999; Halm et al., 2009; Schmidtova et al., 2009). In anoxic basins the redoxclines are well below the photic zone, thus the use of sunlight as the primary source of energy is considered to be negligible in the Baltic Sea or in the Cariaco Basin. From redoxclines of the Black Sea an extremely low-light adapted phototrophic bacterium related to *Chlorobium* sp. was isolated (Overmann et al., 1992). This slowly growing organism is presumably distributed over a relative broad depth range *in situ*, but the overall activity is supposed to be rather low and thus not contributing to a significant part to the chemolithoautotrophic production and sulfide oxidation, especially in the shelf regions of the Black Sea (Manske et al., 2005; Overmann and Manske, 2006; Marschall et al., 2010).

Stable isotopes in microbial ecology

Linking microbial identities with distinct activities has been a challenge for a long time in microbiology. Due to low cultivability of the most environmental microorganisms just a small basis for substantial biochemical and physiological analyses exists (Olsen et al., 1986; Amann et al., 1995; Schut et al., 1997; Meyer-Reil, 2005). The advances in molecular biology and the concept of the full 16S-RNA-cycle approach (Giovannoni et al., 1988; Amann et al., 1990) provide definitively more detailed information about the capacious and still underestimated diversity of the microbiome (Schmidt et al., 1991; Ludwig and Schleifer, 1994; Hugenholtz and Pace, 1996). Despite this progress in identification of organisms down to species-level or below, the known activities cannot be assigned to a certain organism only by phylogenetic analyses.

In recent years different cultivation-independent methods attracted attention in microbial ecology, all of them exploit the incorporation of a labeled substrate in their biomass. Depending on the approach the taxonomical resolution but also the quantification of the substrate incorporation may differ. In the following paragraph different approaches will be presented, with focus on the rRNA-based SIP method.

A method based on radioactive isotopes combines the phylogenetic identification via oligonucleotide probes and the detection of substrate incorporation by microautoradiography. Depending on the utilized probe this approach is designated MICRO-FISH (fluorescence in situ hybridization) (Lee et al., 1999; Ouverney and Fuhrman, 1999) or MICRO-CARD-FISH (catalyzed reporter deposition fluorescence in situ hybridization) (Teira et al., 2004; Herndl et al., 2005). This culture-independent method allows an excellent quantification of the active members of the community as well as an estimation of the amount of incorporated substrate (Nielsen et al., 2000, 2003). The taxonomical resolution depends on the specific probe,

whereas a specific probe design may permit identification down to species level (reviewed in (Wagner et al., 2006; Neufeld et al., 2007b).

The most sensitive approach, which allows tracking even little changes in isotopic composition, is the mass spectrometric analysis of biomarkers, mostly fatty and amino acids (Summons and Powell, 1986; Dobbs et al., 1989; Abraham et al., 1998; Pond et al., 1998). The pronounced high sensitivity facilitates the analyses of natural isotopic content, but also incorporation studies are possible, while even the amount of incorporated substrate can be calculated (Boschker et al., 1998, 2001). The low taxonomical resolution is the major disadvantage of this method, but some fatty acids can be indicative for distinct groups (Neufeld et al., 2007a).

Methods that track the incorporation of stable isotopes in nucleic acids became most important the last years. Despite the disadvantages in quantification, the taxonomical resolution of identified organisms is surpassing high. Employing this method set can provide detailed information about the identity of the involved microorganisms up to the species level or even below. Due to non-targeted employment of this approach new or unexpected insights in microbial biochemical and physiological are possible, shedding new light on ecological processes. In general, the labeled nucleic acids are separated by ultracentrifugation according to their buoyant density and can afterwards be analyzed by different molecular techniques. The first studies employing stable isotope probing (SIP) used DNA as the investigated biomarker (Radajewski et al., 2000; Whitby et al., 2001). Besides the easy handling of DNA this macromolecule provides a very high taxonomical resolution and provides the opportunity for continuative metagenome studies (Radajewski et al., 2003; Dumont et al., 2006; Chen et al., 2008; Kalyuzhnaya et al., 2008). The major disadvantage is the strongly reduced sensitivity compared to all other approaches, leading to relatively long incubation times and proportionally high substrate concentrations (Radajewski et al., 2003).

In the last years the rRNA-based SIP method became more important and more applicable. Despite the obvious disadvantages of RNA-handling this method evolved to a powerful tool in microbial ecology. A major advance of this approach is the enhanced sensitivity compared to DNA-SIP, because of the faster and enhanced synthesis of RNA (Manefield et al., 2002). In general, 16S or 18S rRNA species are investigated; the use of mRNA is theoretical possible, but to my knowledge not applied yet. ^{13}C was found as a powerful isotope for this technique, but first attempts were also made to make use of ^{15}N (Buckley et al., 2007). The RNA-SIP method was described first in 2002 (Manefield et al., 2002), since that time many studies dealing with a couple of pathways were published, e.g.

(Lueders et al., 2004a, 2004b, 2004c; Lu and Conrad, 2005; Egert et al., 2007). It has to be kept in mind that stable isotope probing of nucleic acids is considered to be purely qualitative, no quantitative statements on substrate uptake or abundances of active organisms are possible. Most SIP experiments, based on nucleic acids, were done in the "classical" way, including density gradient centrifugation and subsequent molecular analyses, but alternative ways of analysis exist. For example, MacGregor et al. (2002) developed a sophisticated method, where rRNA can be isolated using capture-oligonucleotides (Mag-SIP) and analyzed further by mass spectrometry. This approach allows tracking even little changes in isotopic content, but the taxonomical resolution is certainly reduced, depending on the applied phylogenetic probe (MacGregor et al., 2006; Miyatake et al., 2009).

Thesis outline

Dark CO_2 fixation is a well-known phenomenon in pelagic redoxclines, which is presumably due to chemolithoautotrophy. To date, the underlying energy-yielding pathways and involved microorganisms are still a mater of debate. Indirect evidence for the contribution of a number of microorganisms *in situ* was provided, but the first direct link between activities and identities was only recently disclosed by MICRO-CARD-FISH analyses by Grote et al. (2008). The authors provided evidence for the contribution of *Epsilonproteobacteria* to a major extent to the chemolithoautotrophic production. However, this method allowed an excellent quantification of active chemolithoautotrophs and the assignment to a defined microbial group, but no information about the diversity of the chemolithoautotrophs was given. Moreover, this approach did not identify the whole chemoautotrophic community; up to 30% of the ^{14}C-positive cells could not be assigned.

Furthermore, previous studies emphasized the high abundance of potential chemolithoautotrophic *Epsilonproteobacteria* throughout a relatively broad depth interval (Lin et al., 2006; Grote et al., 2007; Wakeham et al., 2007), where different redox conditions are present. The distribution of the cluster GD17 constituting the major part of this phylum in the Baltic Sea over the redoxclines does not correlate with the dark CO_2-fixation maxima (Grote et al., 2007). Moreover, only approximately 65% of cells belonging to the GD17 cluster incorporate the [^{14}C]-bicarbonate, leading to the hypothesis that this group is either partially inactive or constituted of several closely related strains with different metabolic capacities, or is even represented by one single, remarkably versatile species.

Hence, the aim of this study was to directly link chemolithoautotrophic activity with the identity of microorganisms. To investigate the identity and diversity of specific CO_2-fixing

groups in sulfidic areas of pelagic redoxclines from the central Baltic Sea and the Black Sea the RNA-based SIP method was applied. Moreover, the mixotrophic and heterotrophic potential of a natural community from a Baltic Sea redoxcline was investigated, focusing the hypothesized metabolic versatility of the GD17 subcluster. The present thesis is organized in three chapters, addressing several aspects of carbon metabolism in pelagic redoxclines in anoxic basins.

1. Linking chemolithoautotrophic activity with the identity of responsible organisms in pelagic redoxclines of the Baltic Sea.
2. Linking chemolithoautotrophic activity with the identity of responsible organisms in pelagic redoxclines of the Black Sea.
3. Investigate the potential mixotrophic capacity of the epsilonproteobacterial strain *Sulfurimonas* sp. GD1 under laboratory conditions and of a microbial community in a Baltic Sea redoxcline.

The first chapter was dedicated to the identification of chemolithoautotrophic assemblages in the sulfidic zone of a pelagic redoxcline from the central Baltic Sea (Landsort Deep). Applying a polyphasic approach, including rRNA-based SIP and mass-spectrometric analyses of biomarkers, the link between activity and identity of chemolithoautotrophs was disclosed.

In the second chapter a similar experiment was conducted for sulfidic waters originating from a pelagic redoxcline of the Black Sea. Applying rRNA-based SIP the diversity of chemolithoautotrophic assemblages at the CO_2 fixation maximum was investigated.

The aim of the third chapter was to investigate the mixotrophic and heterotrophic potential of a chemolithoautotrophy-dominated community in a pelagic redoxcline in the central Baltic Sea. After testing the capability of the strain GD1 to metabolize the model substrate pyruvate, with special emphasis on the destiny of carbon in the metabolism, the pyruvate usage of a natural community was investigated.

Altogether, this thesis underlines the role of *Epsilonproteobacteria* in chemolithoautotrophic production of sulfidic areas within pelagic redoxclines of the central Baltic Sea and the Black Sea. Also direct evidence for the contribution of sulfur-oxidizing *Gammaproteobacteria* to dark CO_2 fixation activities was provided. Albeit no mixotrophic organisms were identified *in situ*, evidence for pyruvate-usage and hence, mixotrophic growth of the *Sulfurimonas* sp. GD1 was provided.

^{13}C-isotope analyses reveal that chemolithoautotrophic *Gamma*- and *Epsilon-proteobacteria* feed a microbial food web in a pelagic redoxcline of the central Baltic Sea (Chapter 1)

1.1 Summary

Marine pelagic redoxclines are zones of high dark CO_2 fixation rates, which can correspond up to 30% of the surface primary production. However, despite this significant contribution to the pelagic carbon cycle, the identity of most chemolithoautotrophic organisms is still unknown. Therefore, the aim of this study was to directly link the dark CO_2 fixation capacity of a pelagic redoxcline in the central Baltic Sea (Landsort Deep) with the identity of the main chemolithoautotrophs involved. Our approach was based on the analysis of natural carbon isotope signatures in fatty acid methyl esters (FAME) and on measurements of CO_2 incorporation in ^{13}C-bicarbonate pulse experiments. The incorporation of ^{13}C into chemolithoautotrophic cells was investigated in samples originating from the CO_2 fixation maximum, as determined by rRNA-based stable isotope probing (RNA-SIP) and FAME analysis after incubation for 24 and 72 h under *in situ* conditions.

Our results demonstrated that fatty acids indicative of *Proteobacteria* were significantly enriched in ^{13}C slightly below the chemocline. RNA-SIP analyses revealed that two different *Gammaproteobacteria* and three closely related *Epsilonproteobacteria* of the *Sulfurimonas* cluster were active dark CO_2-fixing microorganisms, with a time-dependent community shift between these groups. Labelling of *Archaea* was not detectable, but after 72 h of incubation the ^{13}C-label had been transferred to a potentially bacterivorous ciliate related to *Euplotes* sp. Thus, RNA-SIP provided direct evidence for the contribution of chemolithoautotrophic production to the microbial food web in this marine pelagic redoxcline, emphasising the importance of dark CO_2-fixing *Proteobacteria* within this habitat.

1.2 Introduction

All extant forms of life directly or indirectly depend on autotrophic CO_2 fixation. In quantitative terms, CO_2 fixation is the most important biosynthetic process on Earth (Hügler et al., 2003) and it depends on energy deriving from light (photosynthesis) or from exergonic chemical transformations (chemosynthesis). Chemosynthesis is a solely prokaryotic feature requiring inorganic compounds, such as molecular hydrogen and reduced nitrogen (NH_4^+, NO_2^-), sulfur (e.g. H_2S, $S_2O_3^{2-}$), metals (e.g. Fe^{2+}, Mn^{2+}) or carbon (e.g. CO), serving as electron donors (Shively et al., 1998) and predominantly oxygen or nitrate as electron acceptors.

The pelagic redoxcline refers to the gradient in the redox state between the oxic and anoxic interface in stratified aquatic systems (Hallberg, 1972). In these suboxic to sulfidic transition zones, dark CO_2 fixation can be observed; for instance, in the Black Sea (Jørgensen

and Bak, 1991; Sorokin et al., 1995), the Cariaco Basin (Taylor et al., 2001), the Mariager Fjord (Zopfi et al., 2001), hydrothermal vents (Tuttle and Jannasch, 1979) or salt wedges of rivers (Casamayor et al., 2001).

Recent studies in the Black Sea indicated that chemolithoautotrophy within the suboxic to anoxic zones is linked to archaeal and bacterial nitrification (Wuchter et al., 2006; Lam et al., 2007) and bacterial anaerobic ammonium oxidation (Kuypers et al., 2003), respectively. However, the highest dark CO_2 fixation rates have mostly been observed directly at or below the chemocline (zone of uppermost sulfide appearance) for different redoxclines worldwide, where nitrification and anammox should play a minor role. Potentially chemolithoautotrophic *Epsilonproteobacteria* could be involved within these zones, where they may comprise up to 30% of total cell counts (Lin et al., 2006), but, as yet, direct evidence for their chemolithoautotrophic lifestyle is lacking.

The Baltic Sea, as our study site, is among the largest brackish basins of the world, with periodically anoxic conditions in its bottom waters. The Baltic Sea proper comprises a number of deep areas with anoxic bottom waters, of which the Gotland Deep is the largest and the Landsort Deep the deepest. A stable halocline below 60–70 m separates the water column into the upper oxygenated layer and the underlying oxygen-deficient and anoxic/sulfidic layers (Lepland and Stevens, 1998; Neretin et al., 2003). For pelagic Baltic Sea redoxclines, maximal dark CO_2 fixation rates have usually been recorded for sulfidic waters 10–20 m beneath the chemocline (Labrenz et al., 2005), where chemolithoautotrophic bacteria can account for up to 40% of total prokaryotes or approximately $2–5 \times 10^5$ cells ml^{-1} (Jost et al., 2008). Previous stimulation experiments in which inorganic substrates were added to original water samples suggested that an epsilonproteobacterium related to *Sulfurimonas denitrificans* is a key organism for chemolithoautotrophic denitrification in Baltic redoxclines (Labrenz et al., 2005; Brettar et al., 2006) and that it represents a substantial fraction of the prokaryotic community around the chemocline (Grote et al., 2007). However, a direct link between dark CO_2 fixation within sulfidic areas and the identity of the organisms or assemblages involved has never been established for the central Baltic Sea, or for any other comparable marine redoxcline, to date.

Thus, the aim of this study was to address this apparent knowledge gap and to establish a direct link between chemolithoautotrophic activity and microbial identity for the pelagic redoxclines of the Landsort Deep (central Baltic Sea). A biphasic approach was chosen based on the use of ^{13}C-isotope tracers: (1) A depth profile of natural carbon-isotope abundance was recorded using fatty acid methyl ester (FAME) analysis to directly locate the depth at which *in situ* CO_2 incorporation values into bacterial fatty acids were highest; and (2)

^{13}C-bicarbonate incorporation experiments with water samples obtained from these depths were combined with rRNA-SIP to trace the transfer of ^{13}C-label into abundant autotrophs and, potentially, also to organisms of the second trophic level under close to *in situ* conditions.

1.3 Experimental Procedures

1.3.1 Sampling

Samples were taken from onboard the research vessel "R/V Professor A. Penck" during cruise 40/06/16 in the Landsort Deep (station 284; 58°35'N, 18°35'E), in July 2006, using conductivity, temperature, and depth (CTD) (SBE 911plus, Seabird) probes connected with a water-sampling device. Physico-chemical profiles to determine the oxygen, hydrogen sulfide, ammonium, nitrite and nitrate content of the water column were obtained at the sampling location and done as described elsewhere (Grashoff et al., 1983).

1.3.2 *In situ* CO_2 dark fixation

CO_2 fixation rates throughout the redoxcline were determined as described by Steemann Nielsen (1952) with minor modifications as follows: 100 μCi ^{14}C-bicarbonate in anoxic solution (specific activity 53.0 mCi mmol^{-1}, Hartmann Analytic GmbH) was added to 120-ml Winkler bottles containing the collected water samples from different depths of the water column. The bottles were then incubated at *in situ* temperatures for 24 h in the dark, after which the samples were filtered onto 0.2-μm membrane filters, exposed to HCl fumes for 20 min, and counted in a liquid scintillation counter (Packard).

1.3.3 Cellular fatty acid analysis

Fatty acid methyl esters (FAMEs) were analysed in *in situ* water samples and in ^{13}C-labeled samples. Cellular fatty acids were extracted from one filter per depth and incubation time. Gas chromatography/mass spectrometry (GC-MS) analyses were run three times.

For cellular fatty acid analysis, cells in water samples were saponified (15% (w/v) NaOH, 30 min, 100° C), methylated to FAMEs (methanolic HCl, 10 min, 80° C), and extracted [hexane/methyl-tert-butyl ether (1:1, v/v)] as described in detail by Osterhout et al. (1991). FAMEs were analysed on a Hewlett-Packard (HP) 5890A gas chromatograph and were separated on a fused-silica capillary column (25 m × 0.2 mm) with cross-linked 5% phenyl methyl silicone (film thickness 0.33 μm; HP Ultra 2). The computer-controlled parameters were described by Osterhout et al. (1991). The instrument was equipped with a flame ionisation detector and an autosampler (HP 7673). H_2 served as carrier gas.

Gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) of fatty acids was performed on a Finnigan MAT 252 isotope ratio mass spectrometer coupled with a HP 5890 gas chromatograph via a combustion interface. The separation and combustion of fatty acids are described in detail in Abraham and Hesse (2003). Stable isotope composition was expressed in the δ notation with PeeDee belemnite as standard (Craig, 1957). Due to derivatisation of the fatty acids, an additional carbon was introduced, which resulted in alteration of the isotope ratios. Therefore, calculation of the carbon isotope ratio of fatty acids included a correction for the isotope ratio of the methyl moiety to obtain the original isotope ratio of fatty acids using a previously published equation (Goodman and Brenna, 1992; Abrajano Jr. et al., 1994).

Multivariate analysis of fatty acid data was done by cluster analysis using the PRIMER software and following the method of Clarke and Warwick (1998). Normalised values were used for $\delta^{13}C$ and Euclidian distance. Clustering significance was tested according to the ANOSIM algorithm; global R values above 0.75 indicated separation (Clarke and Gorley, 2001).

1.3.4 ^{13}C-incorporation assay

^{13}C-bicarbonate (Campro Scientific) was added anoxically to 610 ml of water samples (four replicates each) taken from depths of 65 m (suboxic water layers), 77 m (CO_2 fixation maximum) and 90 m (sulfidic water below the CO_2 fixation maximum), each at a final concentration of 2 mmol l^{-1}, which was equal to the natural concentration of unlabelled bicarbonate *in situ*. The labelled samples were incubated in the dark at *in situ* temperature and under anoxic conditions. After 24 and 72 h, the bottles were emptied and the water was filtered through Durapore filters (0.2-µm pore size) and then shock-frozen. An unamended sample incubated for 72 h and containing the naturally available 2 mmol of unlabelled bicarbonate l^{-1} served as the ^{12}C-control.

1.3.5 Nucleic acid extraction

Due to the relatively high amount of RNA needed for SIP experiments, three replicates of filters had to be pooled. RNA from filters taken from depths of 77 and 90 m was extracted from frozen filters according to a previously described method (Weinbauer et al., 2002) and using an acidic extraction buffer (50 mmol sodium acetate l^{-1}, 10 mmol EDTA l^{-1}, 2% SDS (w/v), pH 4.2) and extraction-buffer-equilibrated phenol-chloroform (8:1, pH 4.2). The aqueous phase was washed twice with chloroform-isoamylalcohol (24:1) followed by precipitation with 1 volume isopropanol, 0.1 volume of 3 mol sodium acetate l^{-1} and 26 µg glycogen as carrier. The RNA was washed, dissolved and then quantified in a NanoDrop ND-

1000 spectrometer (NanoDrop Technologies). Co-precipitated DNA was removed by digestion with DNase I (Ambion). Prior to isopycnic centrifugation, RNA was quantified using the RiboGreen quantification kit (Molecular Probes).

1.3.6 Isopycnic centrifugation and gradient fractionation

Gradient preparation, isopycnic centrifugation and gradient fractionation were done as described elsewhere (Lueders et al., 2004a), with minor modifications. Each gradient consisted of 5.1 ml CsTFA (approx. 2 g/ml, Amersham), 185 µl formamide and 1 ml gradient buffer (100 mmol Tris-HCl, pH 8.0, l^{-1}, 100 mmol KCl l^{-1}, 1 mmol EDTA l^{-1}) including 500 ng of DNA-free RNA. Prior to centrifugation, the average density of the centrifugation medium was controlled refractometrically and adjusted to an average density of 1.80 g cm^{-3} if necessary. The samples were centrifuged in 5-ml polyallomer quick-seal tubes in a VTI 65.2 vertical rotor (both Beckman) using a Centrikon T-2190 centrifuge (Kontron Instruments). Centrifugation was done at 20°C for > 60 h at 37 000 rpm (125 000 × g_{av}). Gradients were fractionated as described before (Neufeld et al., 2007c) and the density of each collected fraction was measured by determining the refractory index. Subsequently, the RNA was precipitated using isopropanol; the pellet was washed once with 70% ethanol and dissolved in 25 µl of 5 mmol Tris-HCl l^{-1}.

Name	Sequence	Target	E. coli Position	Application	Reference
Ba519f	CAG CMG CCG CGG TAA NW	*Bacteria*	519-535	qPCR	(Stubner, 2002)
Ba907r	CCG TCA ATT CMT TTR AGT T	*Bacteria*	907-925	qPCR	(Stubner, 2002)
com1f	CAG CAG CCG CCG TAA TA	*Bacteria*	519-535	SSCP	(Schwieger and Tebbe, 1998)
com2rpH	CCG TCA ATT CCT TTG AGT TT	*Bacteria*	907-926	SSCP	(Schwieger and Tebbe, 1998)
Ar109f	ACK GCT CAG TAA CAC GT	*Archaea*	109-125	qPCR	(Lueders and Friedrich, 2003)
Ar912rt	GTG CTC CCC CGC CAA TTC CTT TA	*Archaea*	912-934	qPCR	(Lueders and Friedrich, 2003)
Euk1f	CTG GTT GAT CCT GCC AG	*Euk.*	4-20	DGGE	(Diez et al., 2001)
Euk516r-GC	ACC AGA CTT GCC CTC C (GC-clamp)	*Euk.*	563-548	DGGE	(Diez et al., 2001)

Table 1 PCR primer combination used in this chapter.

1.3.7 Quantitative PCR

The 16S rRNA precipitated from 13 fractions was quantified by real-time PCR in a Mx3000P qPCR cycler (Stratagene) using the domain-specific bacterial primer set

Ba519f/Ba907r (Stubner, 2002) and archaeal Ar109f/Ar912rt (Lueders and Friedrich, 2003)(Table 1). For absolute quantification of rRNA molecules, *in vitro* RNA transcripts of cloned bacterial and archaeal full-length 16S rRNA amplicons were used in defined dilution steps from 10^8–10^2 copies per qPCR reaction (Lueders et al., 2004b). The Access One-Step-RT-PCR kit (Promega) was used for RT-qPCR. Each PCR contained 1× PCR buffer, 1mmol $MgSO_4$ l^{-1}, 0.2 μg BSA μl^{-1} (Roche), 0.1 mmol of each dNTP l^{-1}, 0.1× SybrGreen and Rox reference dye, 0.25 μmol of each primer l^{-1} and 3U of the required enzymes AMV reverse transcriptase and *Tfl* DNA-polymerase. Two μl of the dissolved fractionated RNA served as template. PCR was started with reverse transcription followed by an initial denaturation step for 5 min. Amplification was done with a 35-cycle-PCR (30 s 95°C, 30 s 52°C, 30 s 68°C) followed by a terminal elongation step (5 min). After each run, a melting curve between 55 and 95°C was collected to differentiate between specific amplicons and unspecific signals.

1.3.8 rRNA Fingerprinting Analyses

RT-PCR for SSCP analysis of bacterial communities was performed using the primer sets com1f and com2rpH (Schwieger and Tebbe, 1998), which are specific for bacterial 16S rRNA (Table 1). PCR was done as recently described (Labrenz et al., 2007) with slight modifications: Thermal cycling started with reverse transcription, followed by a total of 30 cycles with an elongation temperature at 68°C. The PCR mixture contained 1× PCR buffer, 1mmol $MgSO_4$ l^{-1}, 0.1 mmol of each dNTP l^{-1}, 0.2 μmol of each primer l^{-1} and 5 U of AMV reverse transcriptase and *Tfl* DNA-polymerase. Single-stranded DNA was generated and purified and the SSCP procedure carried out according to (Schwieger and Tebbe, 1998). The gel was silver-stained according to Lee et al. (1996). For further analyses, bands were excised and eluted according to Pöhler et al. (2002).

Microeukaryote communities in water samples from the Landsort Deep were analysed by denaturing gradient gel electrophoresis (DGGE) of the sample representing the CO_2 fixation maximum in one replicate. Three μl of the fractionated RNA from this sample served as template for one-step RT-PCR using the Access Kit (Promega) and the eukaryotic primers Euk1f and Euk516r-GC (Diez et al., 2001) at a final concentration of 0.2 μmol l^{-1} (Table 1). The PCR mixture contained 1× PCR buffer, 1mM $MgSO_4$, 0.1 mmol of each dNTP l^{-1} and 5 U of AMV reverse transcriptase and *Tfl* DNA-polymerase. After reverse transcription, PCR was started with an initial denaturation step at 94°C for 2 min followed by 33 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s and elongation at 68°C for 2 min. The terminal elongation step was done at 68°C for 6 min. The samples were electrophoresed on 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide 37.5/1) with a linear

denaturating gradient of 40–55% using the Ingeny PhorU system. Complete (100%) denaturation was achieved with 7 M urea and 40% deionised formamide. About 800 ng of each PCR product was loaded in each lane. Gels were run for 16 h at 100V at 60°C with 1× TAE as electrophoresis buffer and then stained for 50 min with 1× TAE buffer containing SybrGold. The bands were visualised by UV radiation and excised under illumination with blue light. Excised bands were eluted in nuclease-free water and reamplified using the same primer set without the GC clamp.

The DGGE and SSCP fingerprinting gels were digitalised and the resulting images processed using GelCompar II (Applied Math) based on densitometric curves. After normalisation and background subtraction, the relative contribution of each band to the total band intensities of each lane was calculated.

1.3.9 Sequence analysis

Excised bands were reamplified using the primer systems com1f/com2rpH or Euk1f /Euk516r (without a GC clamp) for SSCP and DGGE, respectively. PCR products were purified using the Nucleospin II kit (Macherey & Nagel). Sequencing was done by JenaGen (Jena, Germany) or Qiagen (Hilden, Germany). Sequence reads were quality checked using the program Seqman (DNAstar). Preliminary estimates of phylogenetic affiliations of the 16S rRNA sequences were obtained by BLAST (Altschul et al., 1997). Alignment and phylogenetic analyses of the obtained sequences were performed using the ARB software package (Ludwig et al., 2004). Sequences for analysis were reduced to unambiguously alignable positions as determined with group-specific filters. Evolutionary distance dendrograms were constructed using neighbor-joining. Bootstrap analyses were performed with 1,000 resamplings.

1.3.10 Nucleotide sequence accession numbers

rRNA gene sequences of induced organisms were deposited in GenBank database under the accession numbers EU673342 – EU 673347

1.4 Results

1.4.1 Biological and physico-chemical characteristics of the water column

The physical and chemical parameters of station 284 are given in Fig. 5. The thermocline, located at a depth between 15 and 25 m, separated the upper, warmer water layer from the cold, so-called winter water (Fig. 5a). Deeper, salinity increased from 5.5‰ at 40 m to 7.3‰ at 60 m. Within this depth interval, the oxygen concentration decreased sharply, from

380 µmol l^{-1} at 40 m to 31.8 µmol l^{-1} at 60 m. Below the chemocline, located at 65 m, the sulfide concentration increased steadily, up to 13.2 µmol l^{-1}, while below 70 m the concentration of ammonium increased to more than 4 µmol l^{-1}. Nitrite was always present at low concentrations but peaked clearly (0.05 µmol l^{-1}) just below the halocline (~60 m). Nitrate also peaked sharply at this depth (~4.5 µmol l^{-1}) and was hardly detectable above or below it (Fig. 5b). Maximum dark CO_2 fixation rates seemed to not directly co-localise with the observed counter-gradients of sulfide, ammonia, nitrate, and oxygen (Fig. 5c). The rates increased from 0.09 µmol l^{-1} d^{-1} above 65 m to a maximum of 0.5 µmol l^{-1} d^{-1} at 77 m. Below 90 m, dark CO_2 fixation rates decreased again, to ~0.2 µmol l^{-1} d^{-1}. Thus, maximum dark CO_2 fixation rates were observed approximately 10 m beneath the depth at which oxygen and nitrate disappeared.

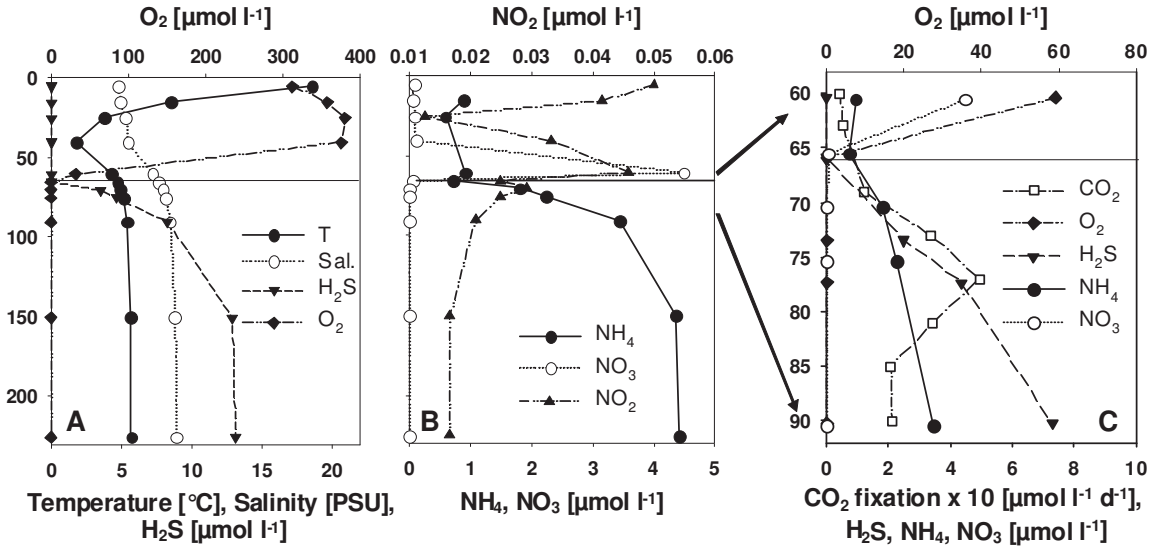

Figure 5 Depth profiles of the Landsort Deep in July 2006.

The line marks the chemocline. (A) Hydrogen sulfide and oxygen concentrations, salinity and temperature. (B) Concentrations of nitrate, nitrite and ammonium. (C) Dark CO_2 fixation rate and a close-up of oxygen, hydrogen sulfide, ammonium and nitrate concentrations at the redoxcline.

1.4.2 FAME analysis

Multivariate analysis using depth and the $\delta^{13}C$ values of biomarkers as parameters allowed the fatty acids to be grouped into two main clusters - a result that was confirmed by one-way ANOSIM (analysis of similarities). A significant division occurred at a distance of 0.8, with a global R-value of 0.776 (p<0.01). Fatty acids with considerable changes in their $\delta^{13}C$ values throughout the water column (C14:0, C16:0, C16:1ω7, C18:1ω7 and C18:1ω9) made up

cluster I (Fig. 6a), whereas cluster II comprised fatty acids with nearly constant isotope contents. Fatty acids of cluster I were relatively ^{13}C-depleted in the upper water layers, but became more enriched in ^{13}C with increasing depth (Fig. 2b). The $\delta^{13}C$ maxima were located above the CO_2 fixation peak at 77 m: for C14:0, the $\delta^{13}C$ maximum of -21.9‰ occurred at 60 m, C16:1ω7 and C18:1ω9 were maximally enriched, with -22.1 and -18.4‰, respectively, at 65 m, and the maxima of C16:0 and C18:1ω7, -23.4 and -19.8‰, respectively, at 70 m. Below, the ^{13}C-isotope content decreased again with increasing depth; however, fatty acids were still enriched in ^{13}C compared to the content in the upper water layer (Fig. 6b).

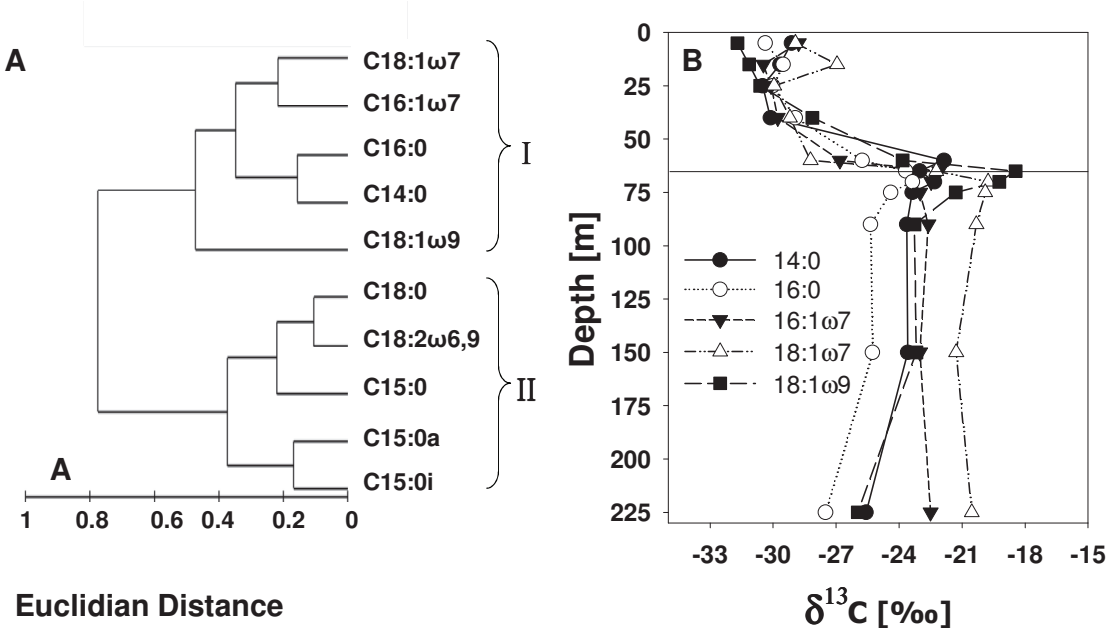

Figure 6 Fatty acid profile of the water column in the Landsort Deep.

(A) Cluster analysis of standardised $\delta^{13}C$ ratios of fatty acids throughout the depth profile at the sampling location. Two main clusters were identified, as indicated by Roman numerals. (B) In situ $\delta^{13}C$ values of the fatty acids grouped in cluster I. These were identified to show clear depth-dependent shifts in $\delta^{13}C$. The $\delta^{13}C$ notation is given in relation to the PDB standard. The line marks the chemocline.

Analyses of ^{13}C-labeled samples originating from the CO_2 fixation maximum showed that those FAMEs enriched in ^{13}C *in situ*, i.e. C14:0, C16:0, C16:1ω7 and C18:1ω7, were also specifically enriched *in vitro*. The highest enrichment was observed for the FAME C16:1ω7, with a $\delta^{13}C$ value of 3000‰, equivalent to an absolute ^{13}C content of 4.5%. This corresponds to a more than four-fold higher ^{13}C content than measured in unlabeled samples. These FAMEs were analogously enriched in ^{13}C in ^{13}C-labeled samples taken from sulfidic water at a depth of 90 m (Fig. 7).

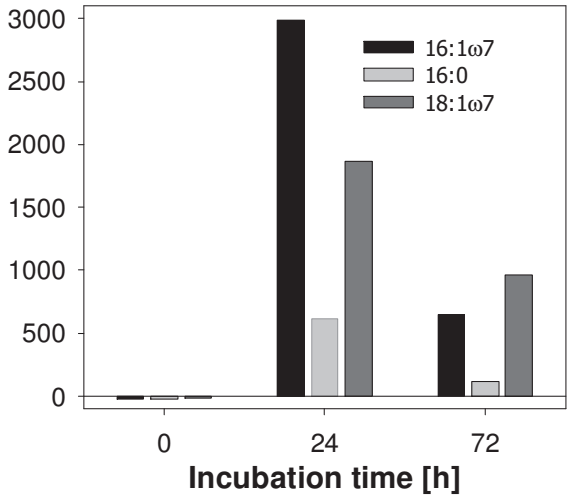

Figure 7 $\delta^{13}C$ values of selected fatty acids from the incubation experiments.

The $\delta^{13}C$ notation is given in relation to the PDB standard. The line marks the chemocline.

1.4.3 Ribosomal RNA separation and fingerprint analyses

The density-resolved distribution of bacterial and archaeal 16S rRNA templates in the respective centrifugation gradients revealed clear differences in the rRNA buoyant density (BD) distribution for those bacterial templates (Fig. 8a) acquired from a depth of 77 m.

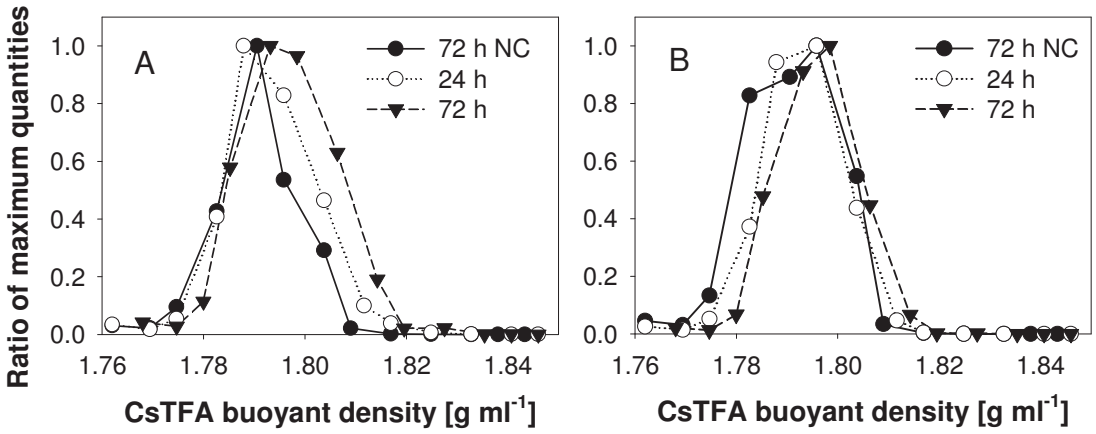

Figure 8 Quantitative distribution of rRNA in CsTFA density gradients.

Ribosomal RNA was extracted from water samples after 24 and 72 h of incubation with ^{13}C-bicarbonate, and after 72 h of incubation without ^{13}C-bicarbonate (NC). (A) Bacterial 16S rRNA. (B) Archaeal 16S rRNA. Domain-specific template distribution within gradient fractions was quantified by quantitative reverse transcription-PCR. Data are given as dimensionless normalisations for reasons of comparability between gradients. Archaeal 16S rRNA amounts were about one order of magnitude lower than those of bacterial 16S rRNA.

Compared to the unamended control, incubation with ^{13}C-bicarbonate caused a time-dependent shift towards heavier BDs for the fractions containing substantial amounts of bacterial rRNA (Fig. 8a). A comparison of the mean BD of the 72-h negative control and that of the ^{13}C-incubation at the same time point showed an enhancement of the latter to 0.004 g cm^{-3}. In contrast to *Bacteria*, no obvious ^{13}C-labelling of rRNA was observed for *Archaea* (Fig. 8b); hence, *Archaea* were not further considered for fingerprint analyses.

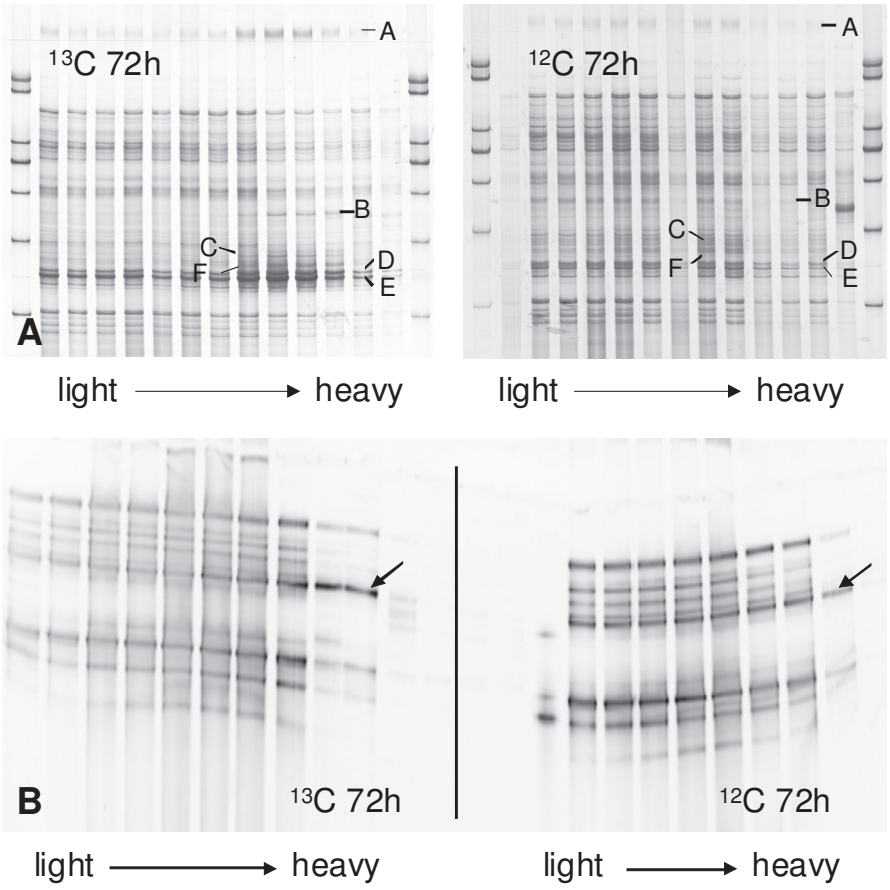

Figure 9 Fingerprints of density-resolved bacterial (A) and eukaryotic (B) SSU rRNA templates from CsTFA density gradient fractions.

The lanes of each gel represent fractions with increasing buoyant densities (BDs) from left to right. Bands identified by sequencing are specified. Relative band intensities were evaluated for all fractions besides the lightest and the heaviest, which usually contain erratic community patterns.

The 16S rRNA single-stranded conformation polymorphism (SSCP) fingerprints done for gradient fractions prepared from a depth of 77 m revealed six different bands specifically enriched in the heavier fractions of the ^{13}C-incubations (Fig. 9a). By gel excision and sequencing, these bands were identified as representing phylogenetic members of *Gamma-* and

Epsilonproteobacteria (Fig. 10). Bands C, D, E and F were closely related to the *Sulfurimonas* subgroup GD17 and to *Sulfurimonas denitrificans*, while bands A and B were related to uncultured organisms and to *Pseudomonas* spp. within the *Gammaproteobacteria*, respectively.

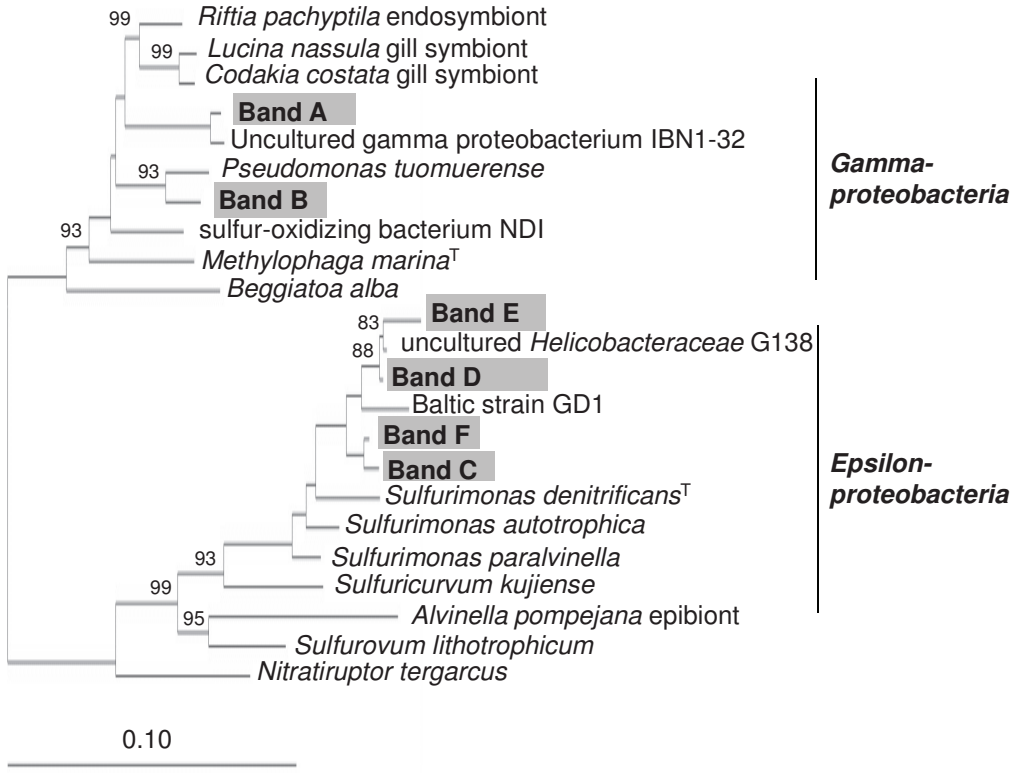

Figure 10 Unrooted phylogenetic tree of bacterial 16S rRNA sequences showing the affiliation of the identified bacterial SSCP bands and their closest relatives within the *Epsilon-* and *Gammaproteobacteria*.

The tree was reconstructed using the neighbour-joining method based on comparison of approximately 400 nucleotides. Only bootstrap values above 70% are shown. Bar, 10 substitutions per 100 nucleotides.

Quantification of the relative intensities for these bands emphasised their clear enrichment in the heavier fractions after 24 and 72 h of ^{13}C-incubation compared to corresponding fractions of the negative control, but also underlined the distinct temporal dynamics of the appearance of these putative key chemoautotrophs (Table 2). Gammaproteobacterial bands A and B showed higher relative enrichment in the heavier fractions after 24 h, while the epsilonproteobacterial bands D and E were relatively stable enriched over time. In contrast, bands C and F only appeared in the heavier fractions after 72 h of incubation with ^{13}C-bicarbonate. SSCP fingerprints from the 90-m gradient fraction yielded analogous results, except for a reduced activity and abundance of gammaproteobacteria (data not shown).

To investigate the potential transfer of ^{13}C-label into the eukaryotic community, 18S rRNA DGGE fingerprinting based on gradient fractions retrieved from a depth of 77 m was also performed. After 72 hours incubation time one band increased in the heavier fractions by 172 % of relative abundance compared to the negative control (Fig. 9b). Sequencing of this band revealed 97% 18S rRNA sequence similarity to the bacterivorous ciliate *Euplotes eurybalinus* (*Ciliophora, Hypotrichia*).

	24 hours		72 hours	
Band	**Maximal enhancement [%] (buoyant density [g cm^{-1}])**		**Maximal enhancement [%] (buoyant density [g cm^{-1}])**	
A	617	(1.826)	331	(1.826)
B	269	(1.826)	38	(1.826)
C	n.d.		69	(1.826)
D	417	(1.834)	540	(1.834)
E	500	(1.813)	390	(1.841)
F	n.d.		989	(1.826)

Table 2 Percentage increase of relative band intensity of induced organisms compared to the respective density fraction of the negative control.

Corresponding fractions as buoyant densities are given in parentheses. n.d. not detectable.

1.5 Discussion

The aim of this study was to directly link chemolithoautotrophic activity with the identity of microorganisms involved in the CO_2 fixation maximum of a Baltic redoxcline located in the sulfidic area. Based on ^{13}C-rRNA-SIP and the analysis of *in situ* FAME isotope ratios, we demonstrated, to our knowledge for the first time, that especially *Gamma-* and *Epsilonproteobacteria* are active anaerobic chemolithoautotrophs within this habitat, and that the ^{13}C-signal is transferred to a phagotrophic eukaryote.

1.5.1 Identification of chemolithoautotrophic microorganisms

Two phylogenetic members of the *Gamma-* and four members of the *Epsilonproteobacteria* were identified as active chemolithoautotrophs based on RNA-SIP, with the earliest labelling effects visible after 24 h of incubation. This time is short enough to exclude secondary effects, such as cross-feeding, which is considered to be a major limitation of SIP (for a review, see Neufeld et al., 2007a). Bacterial 16S rRNA fingerprints demonstrated a time-dependent community shift during the incubation period. After 24 h, the two gammaproteobacterial representatives appeared, as reflected by the increased band intensities

in the heavier fractions. Phylogenetic relatives of both lineages (Fig. 10) were previously found to be associated with environments known to harbor chemolithoautotrophic bacteria, such as anoxic sediments or hydrothermal systems (Bowman et al., 2000; Nakagawa et al., 2005b). Thus, the phylogenetic position of these bacteria suggests their contribution to the sulfur cycle, most probably by the oxidation of reduced sulfur compounds.

The four epsilonproteobacterial representatives were related to the genus *Sulfurimonas*, represented by SSCP bands C–F (Fig. 10). Bands D and E were detected and showed stable ^{13}C-labelling effects after 24 and 72 h of incubation, but C and F only after 72 h (Table 2). The successive appearance of these chemolithoautotrophs during the incubation period could represent an analogous succession at the habitat, where the redoxcline is exposed to vertical and lateral intrusions of different water masses with varying geochemical composition and oxidation status (Lass et al., 2003). Our assumption of a community shift is supported by the fact that both the activity and the abundance of the gammaproteobacteria were reduced 13 m below the CO_2 fixation maximum, based on RNA-SIP analyses. At this depth, the lateral and vertical intrusion of oxygenated or nutrient-rich water masses is less likely.

For the central Baltic Sea, a phylogenetic relative of the epsilonproteobacterium *Sulfurimonas denitrificans* ("uncultured *Helicobacteraceae* G138eps"), detected in bands nearly identical to bands D+E (Fig. 10), was proposed as a key player in autotrophic denitrification in the Gotland Deep (Brettar et al., 2006). Grote et al. (2007) reported that 94% of all *Epsilonproteobacteria* in this habitat were closely related to this key player and consequently grouped them together under the lineage name GD17. GD17 seems to be well adapted to the redoxcline of the central Baltic Sea, based on the prevalence of just a few closely related organisms. However, in general, *Epsilonproteobacteria* have been increasingly recognised as predominantly auto- to mixotrophic organisms that are globally ubiquitous in marine and terrestrial ecosystems (Campbell et al., 2006). A number of studies have verified their significant role, particularly in sulfur-dependent biogeochemical cycles, as demonstrated for deep-sea hydrothermal fields (Nakagawa et al., 2005a; Campbell et al., 2006; Polz et al., 2006), sulfidic cave springs (Engel et al., 2003) and autotrophic episymbiotic associations (Polz et al., 2006; Takai et al., 2006). Molecular studies have confirmed the prevalence and diversity of *Epsilonproteobacteria* in pelagic marine redoxclines of the Black Sea (Vetriani et al., 2003) and the Cariaco Basin (Madrid et al., 2001). In both sites, catalysed reporter deposition-fluorescence in-situ hybridisation (CARD-FISH) analyses using gene probe EPS549 demonstrated maximal epsilonproteobacterial abundances, representing up to 30% of total cell counts in pelagic redoxclines (Lin et al., 2006), usually in zones of remarkable chemoautotrophic activity (Jørgensen et al., 1991; Taylor et al., 2001; Lin et al., 2006). Moreover, *Epsilonproteobacteria*

closely related to the genus *Sulfurimonas* were detected at redoxclines of the Cariaco Basin, Black Sea and Baltic Sea (Madrid et al., 2001; Vetriani et al., 2003; Labrenz et al., 2007). Thus, our data indicate that relatives of *Sulfurimonas* spp. are especially important chemolithoautotrophic organisms in these habitats.

1.5.2 Absence of Archaea

In this study, rRNA levels, which are generally indicative of active organisms, were measured in incubated water samples originating from the maximum CO_2 fixation zone (70 m depth) below the chemocline as well as the sulfidic zone at a depth of 90 m. At both depths, oxygen was not detectable *in situ*, which explains the absence of actively CO_2-fixing aerobic *Crenarchaeota*. These organisms are known to be involved in ammonia-oxidising activities in oxic marine environments (Wuchter et al., 2003; Könneke et al., 2005; Wuchter et al., 2006). In a previous study, close relatives of the microaerophilic, chemolithoautotrophic Crenarchaeon *Candidatus* "Nitrosopumilus maritimus" were found in the lower oxic zone of the Farö Deep, in the central Baltic Sea (Labrenz et al., 2007). However, also due to the low CO_2 fixation rates measured in these depths, an obvious labelling effect in suboxic water layers without prior enrichment seems to be unlikely. Although no chemoautotrophic archaea were detected at or below the CO_2 fixation maximum, these organisms may be active nevertheless and thus contribute to the total chemoautotrophic production above the chemocline.

1.5.3 Activity of chemolithoautotrophs

CsTFA isopycnic centrifugation revealed clear time-dependent labelling effects for the microbial rRNA extracted from ^{13}C-bicarbonate-incubated samples obtained from a depth of 77 m. The presence of fully labelled rRNA, however, was not deducible from the quantitative gradient profiles. Due to the dilution of applied label (2 mM ^{13}C-bicarbonate, with a natural background of ~2 mM unlabelled bicarbonate simultaneously present), this result was expected.

For autotrophically growing organisms, a theoretical shift of the mean BD of about 0.026 was calculated, assuming equilibrium of the isotopes for the applied ratio of $^{13}C/^{12}C$ in total CO_2. The nearly identical amounts of isolated rRNA from the different incubations were indicative of a system in which bacterial growth is nearly in balance with loss-inducing processes such as grazing or phage lyses. Within the whole microbial community, autotrophs represent only a certain fraction, usually 20-40% of the total cell counts, within Baltic pelagic redoxclines (Jost et al., 2008). Based on the assumption that only these autotrophs are responsible for the observed BDs within a dynamic equilibrium, the measured shifts could be explained by a population containing 20% autotrophs having a generation time of 24 h for the

72-h incubation. This would be in agreement with data generated earlier, which combined dark CO_2 fixation measurements with flow cytometric cell sorting (Jost et al., 2008), as well as with the approximately 29% chemolithoautotrophic bacteria calculated directly by microautoradiographic (MICRO)-CARD-FISH (Grote et al., 2008) for a similar redoxcline at the Gotland Basin.

1.5.4 Biomarker isotope ratios

The determination of biomarker isotope ratios is a powerful tool in microbial ecology. Changes in ^{13}C-content are assumed to be a consequence of changes in the origin of carbon sources as well as alterations in the dominating metabolic pathway. In this study, the $\delta^{13}C$ values of different bacterial FAMEs were analysed. Application of multivariate analysis to the data distinguished two distinct groups of FAMEs. In cluster I, the isotope content of C14:0, C16:1ω7, C18:1ω9, C16:0 and C18:1ω7 along the water column changed considerably, with an increase in $\delta^{13}C$ values directly below the oxic-anoxic interface (Fig. 6b). Monounsaturated fatty acids are typical biomarkers for gram-negative bacteria. Li et al. (2007) proposed the monounsaturated fatty acids C16:1ω7 and C18:1ω7 as signature biomarkers for sulfur-oxidising bacteria in H_2S-rich sediments. Saturated fatty acids are widespread among eukaryotes as well as bacteria (Zelles, 1999).

Depth [m]	C14:0	C16:1ω7	C16:0	C18:1ω9	C18:1ω7	$\delta^{13}C_{DIC}$
5	3.4	3.4	-5.1	-13.1	0.9	**-0.78**
15	1.5	-2.4	-2.0	-11.1	7.9	**-0.85**
25	1.3	-1.1	-3.9	-9.2	-2.7	**-0.96**
40	0.0	0.0	0.0	0.0	0.0	**-1.24**
60	29.2	10.6	11.8	6.4	3.6	**-1.87**
65	25.3	27.7	19.6	37.1	25.7	**-2.06**
70	28.0	26.6	20.9	34.4	35.0	**-2.26**
75	24.4	24.8	17.2	26.6	34.8	**-2.46**
90	23.9	26.8	13.8	19.3	33.9	**-3.00**
150	24.8	26.0	14.6	20.3	31.1	**-3.75**
225	17.3	28.0	5.7	8.9	34.1	**-3.80**

Table 3 Estimation of the amount of carbon in FAMES of cluster I throughout the water column originating from CO_2 fixation (%).

40m was chosen as reference depth, where the CO_2 fixation is assumed to be absent. $\delta^{13}C_{DIC}$ was calculated by polynomial regression.

Based on $\delta^{13}C$ dissolved inorganic carbon ($\delta^{13}C_{DIC}$) values, the relative amount of CO_2 fixation was calculated, with 40 m defined as the reference depth at which CO_2 fixation was assumed to be absent. These calculations provided an estimation of the amount of carbon assimilated into specific fatty acids and suggested that up to 37% of the fatty-acid carbon at the redoxcline was derived from autotrophic CO_2 fixation (Table 3). To corroborate this conclusion, the fatty acids of ^{13}C-bicarbonate-labelled samples were analysed. The results showed a similar pattern: some of the fatty acids that were enriched in ^{13}C under *in situ* conditions had extremely high $\delta^{13}C$ values (Fig. 7). Thus, these findings strongly support the results of RNA-SIP analyses and link them to the *in situ* microbial populations.

Neretin et al. (2007) assumed that a decrease in $\delta^{13}C$ values of sulfate-reducing bacteria (SRB)-specific lipids in the Black Sea was a consequence of the chemolithoautotrophic growth of SRB. The major CO_2 fixation pathway used by these bacteria is the acetyl-CoA pathway, which shows strong fractionation of isotopically heavier CO_2 (for a review, see Hayes, 2001). In the Baltic Sea, and according to our SIP results, organisms closely related to *Sulfurimonas denitrificans* are probably the most active CO_2-fixing organisms. *S. denitrificans* is known to use the reverse tricarboxylic cycle (rTCA) for dark CO_2 fixation (Hügler et al., 2005; Takai et al., 2006). This pathway is less discriminating against ^{13}C than either the acetyl-CoA-pathway or photosynthetic CO_2 fixation via the Calvin cycle (Preuß et al., 1989). Accordingly, we conclude that the rTCA cycle is the dominating activity at redoxclines of the central Baltic Sea. The relatively high $\delta^{13}C_{DIC}$ values and the low fractionation during CO_2 fixation should be evident as an increase in $\delta^{13}C$ levels in marker FAMEs.

1.5.5 Potential location of chemolithoautotrophs in the pelagic redoxcline

Cultivation-independent FAME analysis indicated that the CO_2 fixation peak is located above the assumed maximum identified by the radiocarbon method (Figs. 5c, 6b). Grote et al. (2007) determined maximal abundances of GD17 cells directly below the chemocline above the CO_2 fixation maximum, leading the authors to question the contribution of these cells to dark CO_2 fixation. Nevertheless, our data provide evidence that these organisms play a much more important role in CO_2 fixation than suggested by previous studies. Moreover, MICRO-CARD-FISH analyses based on the incorporation of ^{14}C-bicarbonate demonstrated that up to 75% of GD17 cells below the chemocline actively fix CO_2 in the dark (Grote et al., 2008). This discrepancy between the radiocarbon method, GD17 cell numbers and $\delta^{13}C$ values of fatty acids could be due to methodological incompatibilities, but could also reflect the transfer of the ^{13}C-signal within the microbial food web due to viral lysis or predation by eukaryotes.

1.5.6 Microbial foodweb

The fact that in the incubation experiments the ^{13}C-bicarbonate signal was already visible after 72 h in a phagotrophic ciliate, related to *Euplotes eurybalinus*, indicates that chemoautotrophic production had been transferred to the microeukaryotic community. Bacterivorous protists, particularly several ciliate groups, are known to be inhabitants of suboxic and anoxic water layers (Fenchel and Finlay, 1995), and 18S rDNA sequences with 99% similarity to the sequence recovered here have been found in other anoxic marine systems (Stoeck and Epstein, 2003). The transfer of labelled bacterial biomass could have been due to direct protist grazing on chemoautotrophic bacteria, which are a significant component of the bacterioplankton in these water layers. A significant impact of protist predation on the abundance and composition of bacteria has been demonstrated, for example, in the anoxic zone of the Cariaco Basin (Lin et al., 2007). The FAME analysis system is optimised for fatty acids and is unable to detect triterpenes, e.g. tetrahymenol, typical for ciliates. However, it did detect the typical eukaryotic FAME C18:2ω6,9, which was slightly enriched in ^{13}C after incubation of the samples with ^{13}C-bicarbonate. Another likely transfer mechanism of chemoautotrophic bacterial biomass to higher trophic levels is the incorporation of endosymbiotic bacteria. Most known endosymbionts in anaerobic ciliates are methanogens, which are involved in H_2 consumption inside the protists (Fenchel and Finlay, 1995) and are probably chemoautotrophs. Additionally, ectosymbiotic sulfate reducers, which are potentially also chemoautotrophs (when oxidising H_2), are found on the surface of some ciliates (Fenchel and Ramsing, 1992). Future studies should clarify the quantitative significance and the underlying mechanisms of the link between CO_2-fixing bacteria and the heterotrophic protist community.

Diversity of active chemolithoautotrophic prokaryotes in the sulfidic zone of a Black Sea pelagic redoxcline as determined by 16S rRNA-based stable isotope probing (Chapter 2)

2.1 Summary

Marine pelagic redoxclines are characterized by pronounced activities of chemolithoautotrophic microorganisms. As evidenced by the high dark CO_2 fixation rates measured around the oxic-anoxic interface but also in the upper sulfidic zone, the accordant organisms participate in important biogeochemical transformations. Although *Epsilonproteobacteria* have been identified as an important chemoautotrophic group in these environments, detailed species-level information on the identity of actively involved prokaryotes is lacking. In the present study, active chemolithoautotrophic prokaryotic assemblages were identified in the sulfidic zone of a pelagic Black Sea redoxcline by applying rRNA-based stable isotope probing (RNA-SIP) in combination with 16S rRNA single-strand conformation polymorphism (SSCP) analysis and 16S rRNA cloning. The results showed that one single epsilonproteobacterium, affiliated with the genus *Sulfurimonas,* and two different members of the gammaproteobacterial sulfur oxidizer (GSO) cluster were responsible for dark CO_2 fixation activities in the upper sulfidic layer of the Black Sea redoxcline. Phylogenetically, these organisms were closely related to microorganisms, distributed worldwide, that are thought to be key players in denitrification and sulfide oxidation. Together, these findings emphasize the importance of chemolithoautotrophic members of the *Sulfurimonas* and GSO groups in the carbon, nitrogen, and sulfur cycles of oxic-anoxic pelagic transition zones.

2.2 Introduction

Chemolithoautotrophic prokaryotes play an important ecological role in biogeochemical cycles of aquatic habitats. Molecular hydrogen and reduced inorganic compounds, such as nitrogen (NH_4^+, NO_2^-), sulfur (e.g., H_2S, $S_2O_3^{2-}$), and metal species (e.g., Fe^{2+}, Mn^{2+}), as well as carbon compounds (e.g., CO, CH_4) can serve as electron donors for chemoautotrophic bacteria (Shively et al., 1998) whereas oxygen and nitrate mostly serve as electron acceptors. CO_2 dark fixation has been determined in very different habitats, including redoxclines of anoxic marine basins (Sorokin, 1964; Jannasch et al., 1991; Taylor et al., 2001; Jost et al., 2008), marine oxygen minimum zones (Ward et al., 1989), sulfidic caves (Engel et al., 2004), and hydrothermal vents (Karl et al., 1980; Wirsen et al., 1986).

A characteristic of chemolithoautotrophy in pelagic redoxclines of anoxic marine basins is the high fixation rate often observed below the chemocline, defined as the zone in which sulfide is firstly detected. This has been shown for the Black Sea (Jørgensen and Bak, 1991; Sorokin et al., 1995), the Cariaco Basin (Tuttle and Jannasch, 1973b, 1979; Taylor et al., 2001), the Mariager Fjord (Zopfi et al., 2001), and the central Baltic Sea (Gocke, 1989;

Labrenz et al., 2005; Jost et al., 2008). In most cases, the microorganisms responsible for the chemolithoautotrophic activity were identified indirectly; however, more recently, CARD-FISH (catalyzed reporter deposition fluorescence in situ hybridization) combined with microautoradiography (MICRO-CARD-FISH) was successfully used to directly identify *Epsilonproteobacteria* as a quantitatively important chemoautotrophic group in sulfidic zones of the central Baltic Sea and Black Sea (Grote et al., 2008). In another study, rRNA-based stable isotope probing (RNA-SIP) analyses demonstrated the contribution of *Gammaproteobacteria* to dark CO_2 fixation in a redoxcline of the Baltic Sea (Glaubitz et al., 2009).

The aim of this study was to gain more comprehensive information about the diversity of chemolithoautotrophic assemblages in the sulfidic zone of the world's largest anoxic basin, the Black Sea. Using the incubation-dependent rRNA-based SIP method, we identified a single organism of the epsilonproteobacterial *Sulfurimonas* subgroup but also members of the gammaproteobacterial sulfur-oxidizer (GSO) cluster (Lavik et al., 2009) as the drivers of chemoautotrophic production. Close phylogenetic relatives of these Proteobacteria are known to inhabit marine and brackish redoxcline systems worldwide (Madrid et al., 2001; Nakagawa et al., 2005a; Campbell et al., 2006; Stevens and Ulloa, 2008; Glaubitz et al., 2009; Lavik et al., 2009), providing further evidence of their important role in biogeochemical cycles of oxygen-deficient pelagic systems.

2.3 Experimental Procedures

2.3.1 Sampling and *in situ* dark CO_2 fixation

Water samples were obtained in May 2007 during cruise M72 of the research vessel "R/V Meteor" and were collected in the Black Sea (station 7; 43°59.98 N, 32°01.08 E) using free-flow bottles (Hydrobios) attached to a conductivity, temperature, and depth rosette (SBE 911; Seabird). Oxygen and hydrogen sulfide concentrations were determined as described elsewhere (Grashoff et al., 1983). CO_2 fixation rates throughout the redoxcline were determined according to the method of Steemann Nielsen (1952) as described in detail in (Glaubitz et al., 2009) with standard deviations below 10%. Flow cytometric prokaryotic cell counting was done as described previously (Jost et al., 2008) with standard deviations of less than 5%.

2.3.2 ^{13}C-incorporation assay

Incorporation assays were performed for three independent replicates. [^{13}C]-bicarbonate (Eurisotop, Germany) or [^{12}C]-bicarbonate (Merck) was added anoxically to 2 L of

water samples taken from a depth of 145 m to a final concentration of 4 mmol L^{-1}, which was nearly equal to the *in situ* concentration of unlabeled bicarbonate. The treated water samples were incubated in the dark at the *in situ* temperature and under anoxic conditions. After 72 h, the water was filtered through a Durapore filter (0.22 μm pore size) which was eventually shock-frozen. 2.3.3 Nucleic acid extraction, isopycnic centrifugation, and 16S rRNA quantification

DNA-free total RNA was extracted from the frozen samples as previously described (Glaubitz et al., 2009), with minor modifications. After DNase I digestion of co-precipitated DNA the RNA was purified employing another phenol extraction (citrate-buffered phenol:chloroform:isoamylalcohol, 125:24:1, pH 4.2, Fisher Scientific). The aqueous phase was washed once with chloroform:isoamylalcohol (24:1) and precipitated by adding 2 volumes absolute ethanol and 0.5 volumes of 7.5 mol ammonium acetate L^{-1}. Prior to gradient preparation, the washed and dissolved RNA was quantified in a ND-1000 spectrometer (NanoDrop Technologies).

Name	Sequence	Target	E. coli Position	Application	Reference
Ba519f	CAG CMG CCG CGG TAA NW	*Bacteria*	519-535	qPCR	(Stubner, 2002)
Ba907r	CCG TCA ATT CMT TTR AGT T	*Bacteria*	907-925	qPCR	(Stubner, 2002)
com1f	CAG CAG CCG CCG TAA TA	*Bacteria*	519-535	SSCP	(Schwieger and Tebbe, 1998)
com2rpH	CCG TCA ATT CCT TTG AGT TT	*Bacteria*	907-926	SSCP	(Schwieger and Tebbe, 1998)
Ar109f	ACK GCT CAG TAA CAC GT	*Archaea*	109-125	qPCR	(Lueders and Friedrich, 2003)
Ar912rt	GTG CTC CCC CGC CAA TTC CTT TA	*Archaea*	912-934	qPCR	(Lueders and Friedrich, 2003)
27f	AGA GTT TGA TCC TGG CTC AG	*Bacteria*	8-27	Cloning	(Lane, 1991)
1492r	GGT TAC CTT GTT ACG ACT T	*Bacteria.*	1492 -1510	Cloning	(Lane, 1991)
T7	TAA TAC GAC TCA CTA TAG	Vector pSC-A	-	Colony-PCR	-
T3	AAT TAA CCC TCA CTA AAG GG	Vector pSC-A	-	Colony-PCR	-

Table 4 PCR primer combinations used in this study.

Gradient preparation, isopycnic centrifugation, and gradient fractionation were performed as described before (Lueders et al., 2004a), with minor modifications as described

in (Glaubitz et al., 2009): The samples were centrifuged in 5.1-mL Quickseal polyallomer tubes in a VTI 65.2 vertical rotor using a Ultima L-100 XP centrifuge (all Beckman Coulter). Centrifugation was done at 20°C for > 65 h at 35 000 rpm (105 000 × g_{av}). Quantitative RT-PCR (RT-qPCR) of density resolved RNA with the domain-specific primer sets Ba519f/Ba907r (Stubner, 2002) for *Bacteria* and Ar109f/Ar912rt (Lueders and Friedrich, 2003) for *Archaea* (Table 4) was carried out using a one-step reverse-transcription (RT)-PCR kit (Access Kit, Promega) as described previously (Glaubitz et al., 2009).

2.3.4 16S rRNA fingerprinting analyses and cloning

The procedure of SSCP fingerprinting and densitometric analyses of digitalized SSCP-gels was performed as previously described (Glaubitz et al., 2009). Clone libraries were established from PCR products generated from one [^{12}C]-bicarbonate and one [^{13}C]-bicarbonate gradient. The fractions used for this experiment are given in Table 5.

	^{12}C -A	**^{13}C -A**	**^{12}C -B**	**^{13}C -B**
Density (g cm^{-3})	1.782	1.790	1.799	1.816
Clones investigated	311	224	135	103
Different RLFP patterns	43	43	25	15
Coverage (%)	95.8	92.9	92.6	93.2
Shannon-Wiener index	2.86	3.18	2.18	1.78
Evenness	0.76	0.84	0.68	0.66
Chao1	57.1	85.7	33.3	23.6
Abundances (%)				
BS-GSO1	43.1	29.9	63.7	68.0
BS-GSO2	5.1	9.4	3.0	16.5
Other GSO	9.0	18.8	6.7	1.0
Sum of all GSO	**57.2**	**58.0**	**73.3**	**85.4**
Others	**42.8**	**42.0**	**26.7**	**14.6**

Table 5 Descriptive and statistical parameters of all clone libraries.

The Shannon-Wiener and Chao1 indexes were calculated with the FastGroupII online tool (http://biome.sdsu.edu/fastgroup/cal_tools.htm). C-A, clone library of the copy-number maximum; C-B, clone library of the heavier fraction.

RT-PCR was done using the Access Kit (Promega). Nearly-full-length 16S rRNA RT-PCR products were generated using the primers 27f and 1492r (Lane, 1991) (Table 4). The

PCR mixture (15 µl) contained 1× PCR buffer, 1.65 mmol $MgSO_4$ L^{-1}, 200 µmol of each dNTP L^{-1}, 0.2 µg BSA μL^{-1} (Fermentas), 0.7 µmol of each primer L^{-1}, and 1.5 U of AMV reverse transcriptase and of *Tfl* DNA-polymerase (Promega). After a reverse transcription step carried out at 45°C for 45 min, PCR was started with an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and elongation at 68°C for 2 min. The terminal elongation step was done at 68°C for 10 min. The PCR products were directly cloned into the pSC-A vector of the Strataclone system (Stratagene) and subsequently transformed into competent Solopack cells (Stratagene) according to the manufacturer's instructions. Positive clones were selected by blue-white screening. For restriction fragment length polymorphism (RFLP) analyses, the inserts of white clones were amplified using the vector-specific primer combination T7 and T3 (Table 4). PCR mixtures (20 µl) contained 1× PCR buffer, including magnesium, 62.5 µmol of each dNTP L^{-1}, 0.25 µmol of each primer L^{-1}, and 0.75 U of *Taq* polymerase (5Prime). Each of the clones with a specific insert was incubated with 3 U each of the restriction enzymes Hin6I and MspI (Fermentas) in 1× Tango buffer for 150 min at 37°C, followed by a final incubation at 65°C for 20 min. Digested PCR products were analyzed by agarose gel electrophoresis (3% agarose in 1× TAE) and restriction patterns were compared visually. Gene clones with identical patterns were grouped into one operational taxonomic unit (OTU).

Coverage of each of the clone libraries was calculated as follows: $C = 1-(n/N) \times 100$, where N is the total number of clones analyzed, and n the number of OTUs as identified by RFLP analyses (Rappé et al., 1997). The Shannon-Wiener diversity index (H') and the Chao1 index were calculated by using the FastGroup online tool (http://biome.sdsu.edu/fastgroup/cal_tools.htm) as described in (Yu et al., 2006). Maximal diversity was calculated from the number of different OTUs in each clone library: $H_{max} = -[\ln(1/s)]$, with s denoting the total number of species. The Shannon-Wiener diversity index (H') and the maximum diversity index H_{max} were used to calculate the evenness (E) of each clone library, with $E = H'/H_{,max}$. To clarify the differences between two density fractions of each treatment, the enrichment factor was calculated as follows: The relative abundance of each group in the clone library of a heavy fraction was divided by the relative abundance of the same group in the clone library of the fraction in which the maximal copy number occurred. Values below 1 indicated a depletion and values above 1 an enrichment of the accordant organisms in heavier fractions within one density gradient. Enrichment factors equal to 1 indicated that no changes were detectable.

2.3.5 Sequence analyses

Excised bands as well as representative clones were reamplified using the primer systems com1f/com2rpH or T7/T3 for SSCP and clones , respectively (Table 4). PCR products were purified using the Nucleospin II kit (Macherey & Nagel). Sequencing was done by Qiagen (Hilden, Germany) using the primer systems com1f/com2rpH (SSCP bands) and 27f/1492r (clones). Sequence reads were quality checked using the program Seqman (DNAstar). Preliminary estimation of phylogenetic affiliations of the 16S rRNA sequences was done by BLAST (Altschul et al., 1997).

2.3.6 Phylogenetic analyses

The ARB software package was used for the alignment and phylogenetic analyses of the obtained sequences (Ludwig et al., 2004). Sequences for analysis were reduced to unambiguously alignable positions using group-specific filters. For phylogenetic analyses, three different trees were calculated using the algorithms neighbor-joining, parsimony, and maximum-likelihood (Phyml) based on nearly-full-length 16S rRNA sequences (>1350 bp). Shorter sequences were inserted into the reconstructed tree without changing the topology. For neighbor-joining, the Jukes-Cantor correction was applied.

2.3.7 Nucleotide sequence accession numbers

16S rRNA gene sequences of detected organisms were deposited in GenBank database under the accession numbers GU108512-GU108572.

2.4 Results

2.4.1 Physicochemical structure and prokaryotic cell abundances

The chemocline was located at a depth of 135 m. H_2S increased steadily with depth, reaching 9.9 μmol L^{-1} at 160 m. (Fig. 11A). At 126 m, the oxygen concentration was below the detection limit, resulting in a putative anoxic but non-sulfidic zone of about 9 m extent. Below 141 m no nitrate could be detected (G. Lavik, pers. comm.). Two distinct dark CO_2 fixation peaks were detected at the sampling station. At the first, located at a depth of 131 m, a fixation rate of 2.0 μmol L^{-1} d^{-1} was measured, while at the second, at 151 m, the fixation rate was 0.9 μmol L^{-1} d^{-1} (Fig. 11B). Total prokaryotic cell abundances ranged from 3.2×10^5 to 5.0×10^5 cells mL^{-1} (Fig. 11B).

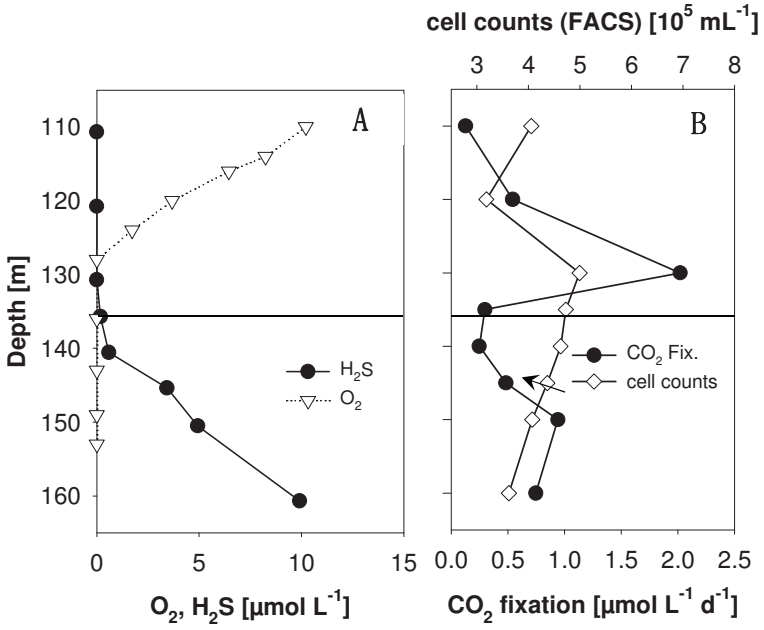

Figure 11 Depth profile of station 7 (43° 59.98 N, 32° 01.08 E) in the Black Sea.

(A) H_2S and O_2 concentrations. The line marks the chemocline. (B) Total cell counts as determined by flow cytometry (FACS), and dark CO_2 fixation. The arrow marks the sampling depth for SIP analyses.

2.4.2 Ribosomal RNA separation and quantification

The rRNA-based SIP analyses were performed using three independent replicates obtained from 145 m depth and incubated with 4 mmol bicarbonate L^{-1} for 72 hours. RT-qPCR specific for bacterial 16S rRNA analyses of three independent replicates were carried out after isopycnic centrifugation and subsequent gradient fractionation and revealed a density shift of the copy-number maximum of 0.0096 g cm^{-3} between the ^{12}C and ^{13}C gradients (p<0.005) (Fig. 12). More than 99% of the rRNA copies were distributed between 1.766 and 1.794 g cm^{-3} in the ^{12}C gradients, and between 1.779 and 1.806 in the ^{13}C gradients. This small difference indicates a partial labeling of the [^{13}C]-bicarbonate incubated bacterial community. The distribution of the [^{12}C]-bicarbonate amendment was very similar to the bacterial copy numbers of the *in situ* community (data not shown).

The absolute archaeal copy numbers were about four orders of magnitude lower than the bacterial copy numbers in the same samples and undetectable after isopycnic centrifugation (data not shown). Hence, the contribution of *Archaea* to chemolithoautotrophic production was assumed to be negligible and not analyzed further.

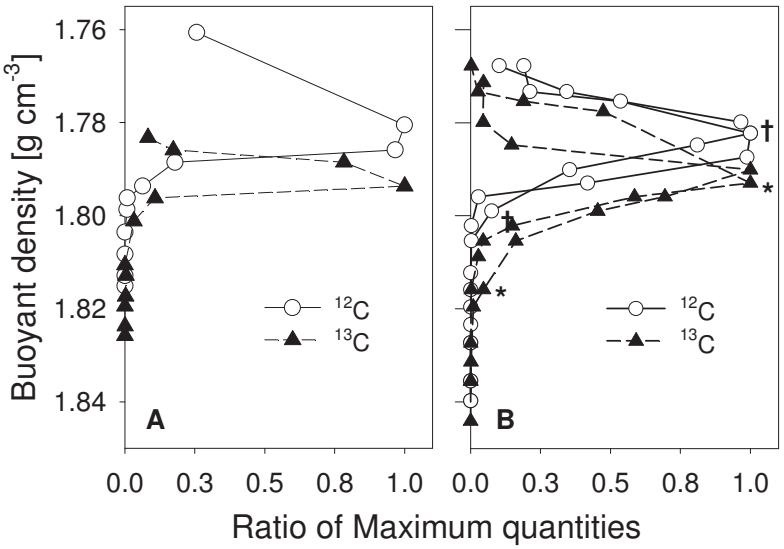

Figure 12 Quantitative distribution of rRNA in CsTFA density gradients after 72 h of incubation with [^{12}C] - and [^{13}C]-bicarbonate.

Domain-specific template distribution within gradient fractions was quantified by quantitative reverse transcription-PCR. Data are given as the dimensionless normalization, to allow comparisons between gradients. (A) Gradients from which SSCP-fingerprints were generated. (B) Replicate gradient preparation. Fractions from which the clone libraries were established are marked by an asterisk (^{13}C) or a cross (^{12}C).

2.4.3 Identification of active chemoautotrophs by 16S rRNA SSCP-fingerprinting

16S rRNA SSCP fingerprinting of the two density gradients revealed that three different bands, named A12-1, A12-2, and A12-3, were affected by the dark [^{13}C]-bicarbonate incubation (Fig. 13A,B). Based on comparisons of their relative intensities, the three bands showed a clear enhancement but also a shift towards heavier fractions in the ^{13}C gradient (Fig. 13C, D). These bands were also detected in a gradient prepared from an unamended *in situ* water sample, but showed minor changes in relative intensity throughout the density gradient (Fig. 14). The sequences determined for bands A12-1 and A12-3 were phylogenetically affiliated with the GSO group, the one in band A12-2 with the epsilonproteobacterial *Sulfurimonas* cluster (Fig. 15). Thus, based on SSCP analyses, the identified organisms were embedded in putative chemoautotrophic clusters within the *Proteobacteria* (Fig. 15). By contrast, the relative intensity of a putative heterotrophic *Pseudoalteromonas* band (A12-4) showed a clear decrease towards heavier fractions in the ^{13}C gradient (Fig. 13C, D).

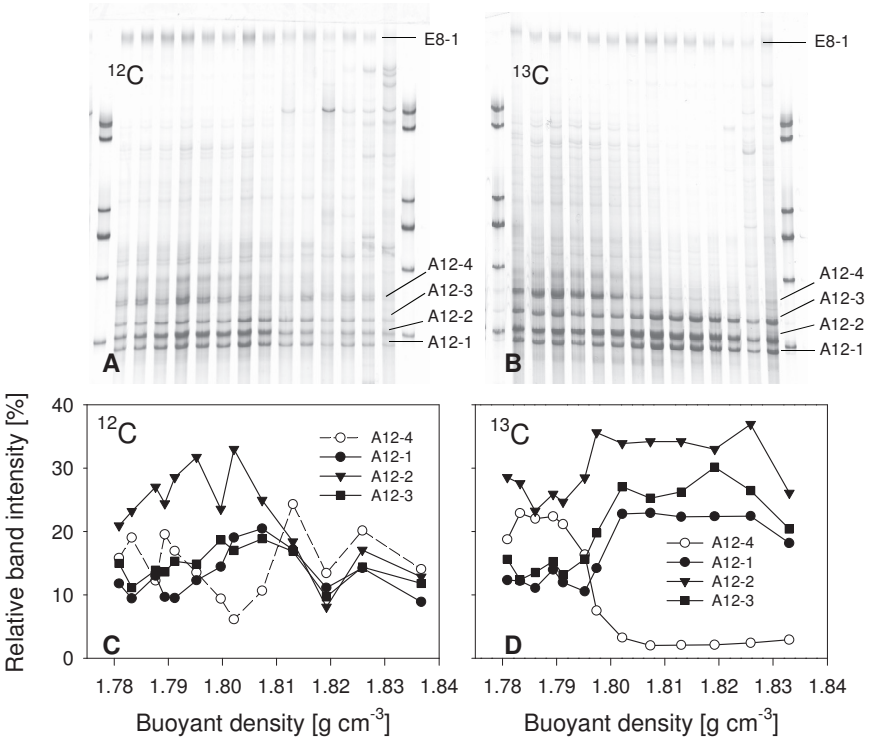

Figure 13 SSCP fingerprints of density-resolved bacterial SSU-RNA templates from CsTFA density gradient fractions of the (A) [^{12}C] - and (B) [^{13}C]-bicarbonate incubation experiments.

The lanes of each gel represent fractions with increasing buoyant densities (BD), from left to right. Bands identified by sequencing are marked. (C) [^{12}C]- and (D) [^{13}C]-bicarbonate-based results of the densitometric analyses of selected bands: Relative band intensities (in percent) are plotted against the respective buoyant densities (in g cm^{-3}). Open circles represent the distribution of a band affiliated with a Pseudoalteromonas species (A12-4) across the density gradient. Closed symbols mark sequences belonging to potentially chemoautotrophic organisms (see text).

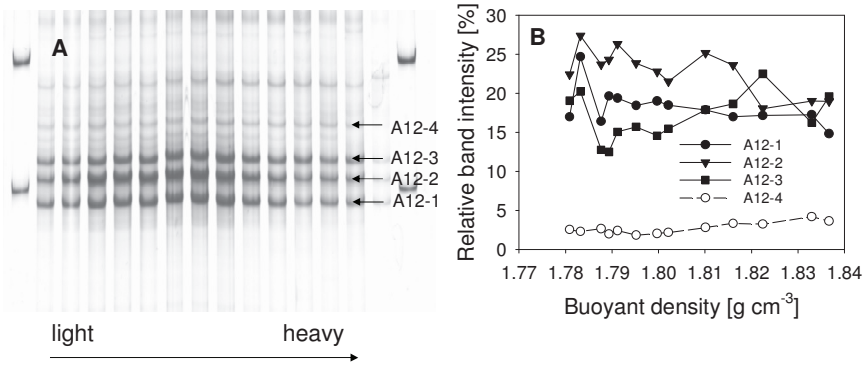

Figure 14 (A) Detail of SSCP fingerprints of density-resolved bacterial SSU-RNA templates from CsTFA density gradient fractions of the *in situ* community in 145 m depth.

The lanes of each gel represent fractions with increasing buoyant densities (BD) from left to right. Bands identified by sequencing are marked. (B) Results of the densitometric analyses of selected bands: Relative band intensities in percent are plotted against the respective buoyant densities in g cm^{-3}. Open circles represent the distribution of a band affiliated to a Pseudoalteromonas species (A12-4) across the density gradient. The closed symbols mark sequences belonging to potentially chemoautotrophic organisms (see text).

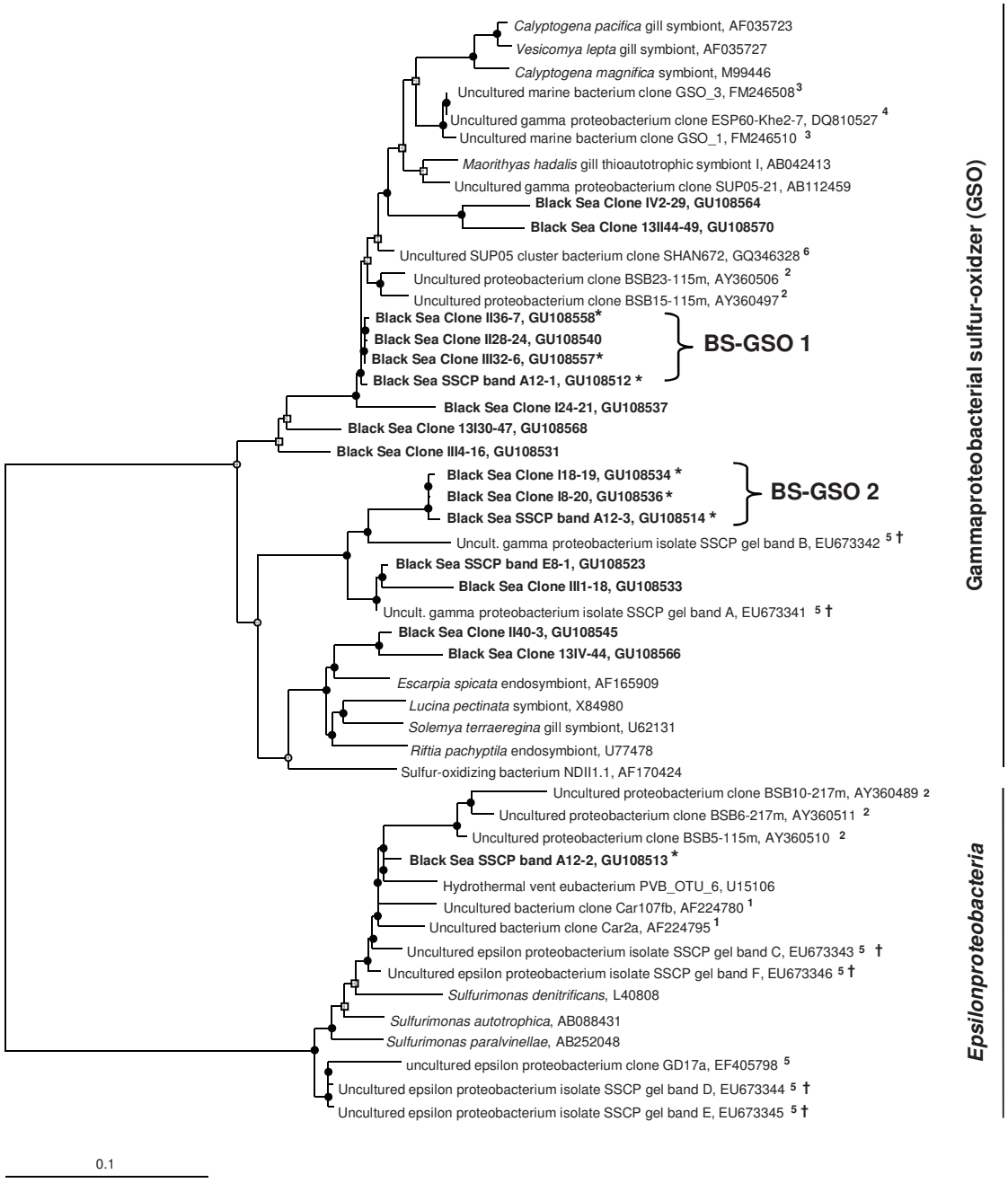

Figure 15 Figure 4 Unrooted maximum likelihood tree of sequences generated in this study that are affiliated phylogenetically with the *Epsilonproteobacteria* and sulfur-oxidizing *Gammaproteobacteria*. Bold letters denote sequences generated in this study. Symbols used in this figure: ● Validation of subtree by neighbor-joining and parsimony, □ Validation of subtree by parsimony, ○ Validation of subtree by neighbor-joining, * Identified as chemolithoautotroph in this study, † Identified as chemolithoautotroph in pelagic redoxclines in previous studies, [1] Detected in a Cariaco Basin redoxcline (Madrid et al., 2001), [2] Detected in a Black Sea redoxcline (Vetriani et al., 2003), [3] Detected in a oxygen minimum zone of the African shelf (Lavik et al., 2009), [4] Detected in a oxygen minimum zone of the eastern tropical South Pacific (Stevens & Ulloa, 2008), [5] Detected in a central Baltic Sea redoxcline (Brettar et al., 2006; Grote et al., 2007; Glaubitz et al., 2009), [6] Based on metagenome analyses from a Saanich Inlet fjord oxygen minimum zone (Walsh et al., 2009). Bar denotes 10 substitutions per 100 nucleotides.

2.4.4 Identification of active chemoautotrophs by 16S rRNA cloning

To investigate the bacterial community structure in greater detail, 16S rRNA clone libraries were established from the copy-number maxima (^{12}C-A and ^{13}C-A) and from heavier fractions (^{12}C-B and ^{13}C-B) (Fig. 12B). According to the descriptive statistical parameters of these clone libraries, coverage was above 90%, indicating that the major part of the diversity was detected by this approach (Table 5). In general, both the Shannon-Wiener index and the evenness factor demonstrated that the 16S rRNA clone libraries of the copy-number maximum were more diverse and more even than those of the heavier fractions (Table 5). All 16S rRNA clone libraries included *Gammaproteobacteria* of the *Alteromonadales*, *Pseudomonadales, Chromatiales, Oceanospirillales, Methylococcales,* and representatives of the GSO group, as well as members of the *Alphaproteobacteria* and the *Planctomycetales* (for a detailed list of all sequenced clones see Appendix I).

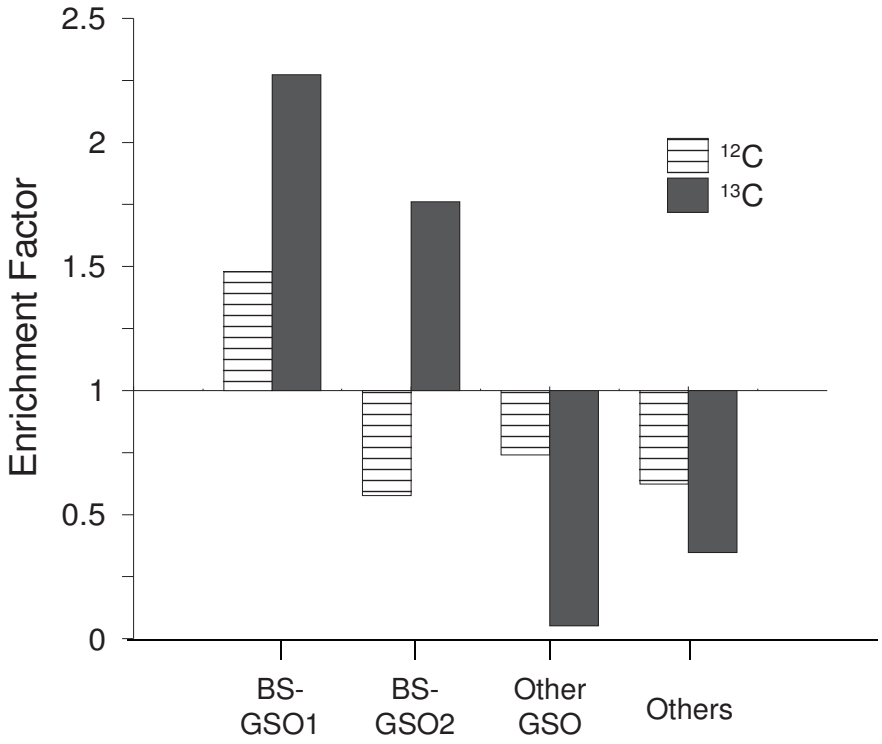

Figure 16 Relative changes in the contribution of selected clones to the composition of the clone libraries.

The dimensionless enrichment factor was calculated by dividing the relative abundance of a clone in the clone library of heavier fractions by the relative abundance of the respective clones in the clone library of the copy-number maximum. Values below 1 indicate depletion and values above 1 indicate enrichment of the particular group in the heavier fractions.

The ^{12}C and ^{13}C 16S rRNA clone libraries of the copy-number maxima were dominated by several members of the GSO group (Table 4), with BS-GSO1 and BS-GSO2

being most abundant. Phylogenetically, the gammaproteobacterial sequences deriving from clone and SSCP analyses were very similar (Fig. 15). A comparison of the ^{12}C and ^{13}C clone libraries from the heavier fractions (Fig. 12) showed that the BS-GSO1 and especially the BS-GSO2 cluster were more abundant in the ^{13}C clone library (Fig. 16). About 99% of all GSO sequences in this library were affiliated with one of these clusters. Other GSO sequences were hardly detectable in the ^{13}C-fraction, indicating that these organisms did not incorporate heavy bicarbonate. Unexpectedly, epsilonproteobacterial clones could not be generated by any of the 16S rRNA clone libraries.

2.5 Discussion

2.5.1 Chemolithoautrophic assemblages in pelagic redoxclines

The aim of this study was to directly link dark CO_2 fixation with the identity of the responsible microbial assemblages in a sulfidic CO_2 fixation maximum of a Black Sea pelagic redoxcline. Using rRNA-SIP and SSCP fingerprinting, we were able to attribute chemolithoautotrophic production to one epsilonproteobacterium affiliated to the genus *Sulfurimonas* and two populations related to the GSO cluster, with clone libraries confirming the role of GSO for the investigated pelagic Black Sea redoxcline.

Chemosynthesis in sulfidic areas of Black Sea pelagic redoxclines is a well known phenomenon (Sorokin, 1964; Jørgensen et al., 1991; Jost et al., 2010). In recent years, several potentially chemolithoautotrophic bacterial organisms or groups have been identified in the Black Sea. For instance, using terminal restriction fragment length polymorphism analyses in combination with 16S rRNA gene cloning, (Vetriani et al., 2003) identified *Epsilon-* as well as *Gammaproteobacteria* below the chemocline. Phylogenetically, these bacteria are closely related to the chemolithoautotrophic epsilonproteobacterium SSCP band A12-2 and the BS-GSO1 cluster of the present study (Fig. 15). Thus, these proteobacterial groups could be widely involved in chemolithoautotrophy in the Black Sea.

Epsilon- and *Gammaproteobacteria* have been quantified in other studies using class-specific gene probes, and cellular abundances of approximately 20 and 6%, respectively, were determined for zones below the chemocline in the Black Sea (Lin et al., 2006; Wakeham et al., 2007). Using CARD-FISH, (Grote et al., 2008) detected epsilonproteobacterial abundances of 11–35% at comparable physicochemical depths. By combining CARD-FISH with microautoradiography (MICRO-CARD-FISH), these authors demonstrated *in situ* the important role of *Epsilonproteobacteria* in chemolithoautotrophy, with 24–100% of all

chemolithoautotrophs phylogenetically belonging to the *Epsilonproteobacteria*. However, specific organisms were not identifiable by these analyses and the rRNA-SIP approach of the present study indicated that the diversity of chemolithoautotrophic *Epsilonproteobacteria* in Black Sea redoxclines can be low, and potentially reduced to only one representative related to the genus *Sulfurimonas*.

A similar phenomenon was observed in a central Baltic Sea redoxcline, where the cellular abundance of the specific *Sulfurimonas* subcluster GD17 may account for 15% (Grote et al., 2007) or occasionally even as high as 30% (unpublished data) of the total cell counts. Subcluster GD17 is closely related to *Sulfurimonas denitrificans* and has been proposed as the dominant player within the sulfur and nitrogen cycle of the central Baltic Sea (Brettar et al., 2006; Grote et al., 2008). Thus, the abundance of a single chemolithoautotrophic epsilonproteobacterial taxon may well be characteristic of pelagic redoxclines of the Black Sea and the central Baltic Sea. Currently, however, this conclusion is based only on random analyses and needs further investigation; nonetheless, it is certainly possible that the presence of unusual or even extreme nutrient or redox conditions in pelagic redoxclines favors specific and well-adapted members of the genus *Sulfurimonas*.

In the above-mentioned MICRO-CARD-FISH study, only *Epsilon-* rather than *Epsilon-* and *Gammaproteobacteria*-specific gene probes were used to quantify chemolithoautotrophic assemblages in sulfidic zones of Black Sea redoxclines (Grote et al., 2008). In this study and in similar pelagic redoxclines, besides *Epsilonproteobacteria* no chemolithoautotrophs other than *Gammaproteobacteria* were detected (Glaubitz et al., 2009). By simply subtracting chemolithoautotrophic epsilonproteobacterial cell numbers from the total number of dark CO_2 fixing cells in the study by (Grote et al., 2008), the contribution of *Gammaproteobacteria* to dark CO_2 fixation can be estimated, resulting in proportions of usually well below 20% on the cellular level. This estimate is in accordance with the FISH data of (Lin et al., 2006), who determined total gammaproteobacterial abundances of 2–6% of the total prokaryotic community in the sulfidic zone of a Black Sea redoxcline. Overall, in the present study, GSO members were found to be more diverse than the *Epsilonproteobacteria*, but only two closely related representatives showed chemolithoautotrophic activity (Figs. 15, 16). One organism, identified by 16S rRNA SSCP (band E8-1, see Fig. 13A,B) and cloning (OTU 18, see Appendix I) and nearly identical to gammaproteobacterium band A, previously shown to be chemolithoautotrophic in the Baltic Sea (Glaubitz et al., 2009), did not incorporate [^{13}C]-bicarbonate in the Black Sea sample. By SSCP and cloning, this organism was determined to be abundant, but its physiological and ecological role in the Black Sea redoxcline is as yet unclear. The co-existence of putative chemolithoautotrophic *Epsilonproteobacteria* and members

of the GSO cluster is presumably reasonable in the different adaptation strategies. The low diversity of epsilonproteobacterial autotrophs and the broad depth distribution suggests a versatile and generalist lifestyle as hypothesized by (Grote et al., 2007, 2008), whereas the considerably enhanced diversity, but reduced activity of GSO might indicate more specialized ecological niches at the species-level.

Despite a potentially lower abundance of chemolithoautotrophic GSO in Black Sea redoxclines, GSO members are in general widely distributed and have been detected in similar habitats as *Epsilonproteobacteria*, such as hydrothermal vents (Ruby et al., 1981), oxygen minimum zones (Stevens and Ulloa, 2008; Walsh et al., 2009), and pelagic redoxclines of the Cariaco Basin (Madrid et al., 2001) and central Baltic Sea (Labrenz et al., 2007). Recently, members of this cluster, which are phylogenetically closely related to the cluster BS-GSO1, were shown to be involved in sulfide oxidation in an oxygen-minimum zone in the Namibia upwelling system (Lavik et al., 2009). There, the abundances of these organisms coincided with the occurrence of sulfide, the formation of colloidal sulfur, and denitrification, which emphasizes the impact of these bacteria on important biogeochemical cycles.

Indirect evidence for the chemolithoautotrophic growth of symbiotic GSO has accumulated from genome and proteome analyses, as well as from the $\delta^{13}C$ values of host organisms, and from enzyme activities (Karl et al., 1980; Felbeck, 1981; Markert et al., 2007; Nakagawa and Takai, 2008; Robidart et al., 2008; Walsh et al., 2009). With the experimental setup in the present study, we were not able to distinguish between free-living and symbiotic lifestyles of the identified GSO, but most metazoans that harbor chemolithoautotrophic endo- or ectosymbionts live in the benthos (reviewed by Stewart et al., 2005) and the probability of occurrence of these hosts in the pelagic, sulfidic habitat is rather low. A similar approach was used to demonstrate the transfer of chemolithoautotrophically fixed ^{13}C-bicarbonate to *Euplotes* sp. (Glaubitz et al., 2009), but a potential symbiotic relationship, conceivable with unicellular free-living eukaryotes, has not been demonstrated thus far.

2.5.2 Methodological considerations

One major point of criticism concerning isotope labeling experiments is related to secondary effects, cross-feeding, and over-labeling of the microbial community (reviewed in Neufeld et al., 2007a), all of which have to be taken into consideration in correctly interpreting the obtained results. In our experiments, the probability of the ^{13}C tracer signal being transferred from lysed autotrophic bacteria to heterotrophic prokaryotes that feed on dissolved or particulate organic matter was considered to be low. Compared to a previously described signal transfer from chemolithoautotrophs to the potential grazer *Euplotes* sp.

(Glaubitz et al., 2009), carbon-source fluxes between bacteria should be negligible due to dilution effects and fractionation within the individual biosynthetic processes. The distribution of ^{12}C-labeled 16S rRNA copies in the density gradients of our study was comparable to that in previously described SIP experiments, whereas the banding of the ^{13}C-labeled rRNA species was indicative of ^{13}C isotope enrichment, but to a lesser extent than expected for completely labeled rRNA species, which results usually in a shift of the copy number maximum of approximately 0.04 g cm^{-3} CsTFA (Lueders et al., 2004a). The enhanced abundance of the BS-GSO1 cluster in the ^{12}C-B library can be explained by the distribution of the specific 16S rRNA copies in the density gradient. The maximal relative intensity of this organism in the ^{12}C-SSCP gel was detected at 1.807 g cm^{-3}, which is heavier than the fraction, the clone library originates from. Using 16S rRNA clone libraries and SSCP fingerprints, we detected also typical heterotrophs, such as *Pseudoalteromonas* spp. and *Alteromonas* spp. (Appendix I), which were less abundant in the heavier fractions of the [^{13}C]-bicarbonate labeled samples (Fig. 13C, D and Fig. 16), implying that in our SIP experiments secondary effects such as cross-feeding can be neglected. Thus, we conclude that the bulk of the observed density shifts were due to the incorporation of [^{13}C]-bicarbonate into chemolithoautotrophs and that other factors, for example, anaplerotic reactions and cross-feeding, accounted for an insignificant portion of the observed effects.

The analysis of band intensities and clone frequencies has to be considered as semi-quantitative due to PCR and cloning biases, differential amount of rRNA operons and copies per cell. The dominance of the GSO and the *Epsilonproteobacteria* in the SSCP gel is presumably due to methological biases, thus potentially overestimated. Comparing fingerprints of the augmented samples with that from the negative control a stimulation of the chemoautotrophs was visible. However, whether it was due to the bicarbonate addition or to potentially introduced electron acceptors cannot be elucidated yet. Despite the laborious procedure of SIP we analyzed three replicate density gradients by qPCR. This effort provided evidence for the practicability and reproducibility of this approach, but no additional information was achieved. The shift of the copy number maximum toward higher buoyant densities was clear in all replicates. For powerful statistical analyses more gradients have to be investigated in detail, but this was not practicable for this study. Hence, the SIP method should be considered as a purely qualitative, non-quantitative approach.

Although we detected an abundant SSCP-band affiliated to the *Sulfurimonas* cluster, the accordant 16S rRNA sequence was absent in our clone libraries. This was unexpected because the primers we used (27f/1492r) are supposed to be universal (Lane, 1991), and in several habitats *Sulfurimonas* spp. were successfully detected using this primer combination already

(Engel et al., 2003; Nakagawa et al., 2005a; Grote et al., 2007). However, concerning a previous Black Sea study the reverse primer 1517R (Vetriani et al., 2003) was used which holds two different bases at the 5'-position compared to 1492r. This could explain that we were unable to detect *Epsilonproteobacteria* based on cloning analogously.

In conclusion, this study provides evidence that chemolithoautotrophic assemblages in marine pelagic redoxclines consist of phylogenetically very similar *Epsilon-* and *Gammaproteobacteria*. This is an indication that these organisms are well adapted to comparable physicogeochemical conditions within such habitats, underlining the importance of *Proteobacteria* in carbon, nitrogen, and sulfur cycles.

On the role of pyruvate as carbon source in a chemoautotrophy-dominated microbial community in a pelagic redoxcline of the central Baltic Sea (Chapter 3)

3.1 Summary

The epsilonproteobacterial subgroup GD17 dominates the chemolithoautotrophic production at redoxclines in the central Baltic Sea. Representatives of this cluster are distributed over a relatively broad depth interval covering alternating redox- and nutrient conditions with maximal 65% of these cells actively fixing CO_2. An isolated representative, the strain *Sulfurimonas* sp. GD1, a potential key player in autotrophic denitrification, is supposed to exhibit a potentially high metabolic diversity, leading to the hypothesis that members of the GD17 group may fulfil more functions in aquatic habitats as hitherto presumed.

The aim of this study was to discover a potential mixotrophic lifestyle of GD1 by using pyruvate as a model substrate applying a polyphasic approach including radiocarbon measurements, gas chromatography-combustion-isotope ratio-mass spectrometry (GC-C-IRMS) and RNA-SIP. In order to investigate the heterotrophic or mixotrophic potential of a natural community, originating from the sulfidic zone of a pelagic redoxcline, the pyruvate utilization was additionally investigated by applying rRNA-SIP.

Our results demonstrated that by RNA-SIP analyses a successful labeling of nucleic acids could not be detected for GD1 as well as environmental samples; whereas in both experiments an uptake of radioactive labeled substrate was present. Fractionation of GD1 cells revealed that approximately 85 % of the signal could be recovered in lipid- and protein-containing fractions. These finding were supported by mass spectrometric analyses, revealing an absolute ^{13}C content of up to 30% in amino acids. These findings are questioning the practicability of the RNA-SIP technology for identifying pyruvate-assimilating GD1 cells. Transferring these data to the identification of pyruvate-using prokaryotes on single-cell level the application of MICRO-CARD-FISH protocols could be more feasible because of their independence of the fate of carbon in the cell metabolism. Nonetheless, our results provide useful insights into the metabolism of GD1, proving a mixotrophic potential at least at laboratory conditions.

3.2 Introduction

Chemolithoautotrophy is a well known phenomenon in different habitats like pelagic redoxclines (Tuttle and Jannasch, 1979; Jannasch et al., 1991; Taylor et al., 2001; Brettar et al., 2006; Grote et al., 2008; Glaubitz et al., 2009), hydrothermal vents (Distel and Cavanaugh, 1994; Scott et al., 1999; Campbell et al., 2003; Robinson et al., 2003; Campbell and Cary, 2004), and even soils (Selesi et al., 2005), or aquifers (Kellermann, 2008). In recent years the knowledge about the identity of chemolithoautotrophic organisms rapidly increased for

pelagic redoxclines of the central Baltic Sea and the Black Sea. The impact of chemolithoautotrophic *Epsilonproteobacteria* was disclosed using MICRO-CARD-FISH and rRNA-based SIP (Grote et al., 2008; Glaubitz et al., 2009). Interestingly, for pelagic redoxclines of the Baltic Sea it has been shown that chemoautotrophic *Epsilonproteobacteria* dominated by the *Sulfurimonas* subgroup GD17 may account for up to 73% of the dark CO_2 fixing cells in a Baltic Sea redoxcline with 65% of the present GD17 cells actively fixing CO_2. This discrepancy was discussed as a metabolic inactivity or even a potential heterotrophy (Grote et al., 2008), but may also be substantiated by an alternating distribution of metabolically divers, but phylogenetically closely related members of this cluster.

The term "chemolithoautotrophy" does not only describe the ability to assimilate inorganic carbon, but also the principal source of energy as well as the involved reducing agents. CO_2 fixation is an energy-demanding process, which is achieved by using the sunlight in photoautotrophs like *Cyanobacteria*, green and red sulfur bacteria or higher plants. In chemolithoautotrophs the energy demand is satisfied by transfer of electrons from reduced inorganic components like sulfide to acceptors like oxygen or nitrate (Lees, 1960; Schlegel, 1960). The required energy for one newly synthesized triosephosphate may differ, depending on the respective CO_2-fixation pathway. The Calvin-Cycle requires for each triosephosphate approximately 9 ATP and 6 $NADPH_2$ (reviewed in Shively et al., 1998); in contrast the fixation via the reverse tricarboxylic acid cycle demands 12 reduction equivalents and 5 ATP (Evans et al., 1966; Campbell and Cary, 2004).

In energy- and nutrient-depleted systems like the pelagic redoxclines in oligotrophic marine systems the usage of low-molecular organic compounds as an additional carbon source by obligate chemolithoautotrophs can be advantageous. Numerous studies on isolated bacterial species revealed that even obligate chemolithoautotrophs can assimilate organic compounds to a certain extent; up to 10% or occasionally even up to 20% of the total cell carbon can derive from low-molecular substrate as acetate, pyruvate or amino acids (Taylor et al., 1971; Matin, 1978; Fuchs et al., 1980a, 1980b; Jannasch et al., 1991) and may enhance cell yield as well as denitrification rates (Cardoso et al., 2006).

Recently *Sulfurimonas* sp. GD1 as a member of the epsilonproteobacterial subgroup GD17 was isolated, acting as a potential key player in autotrophic denitrification in central Baltic Sea redoxclines. From genome analyses as well as autoecological studies is known that this organism, closely related to *Sulfurimonas denitrificans*, has the potential for a distinct metabolic versatility (Grote, 2009). The aim of this study was to determine the mixotrophic potential of GD1, choosing pyruvate as model substrate for DOC-uptake in laboratory experiments. We applied a polyphasic approach including mass spectrometric analyses of

amino and fatty acids and 16S rRNA-based stable isotope method (RNA-SIP) to investigate the fate of the pyruvate-derived carbon in the strain GD1. Moreover, the pyruvate uptake of a natural, chemolithoautotrophy-dominated community was monitored by RNA-SIP analyses. Our results demonstrated that GD1 incorporated pyruvate nearly exclusive into fatty and amino acids. Neither for the strain GD1 nor the natural community a sufficient labeling of nucleic acids was detected, though an apparent uptake of this compound was present. Nonetheless, our results provide detailed into the carbon metabolism of the model strain GD1, underlining the hypothesized ecological significance of this potential key player in pelagic redoxclines of the central Baltic Sea.

3.3 Experimental procedures

3.3.1 Incorporation of radioactive pyruvate by GD1

Incorporation experiments were conducted in glass test tubes, containing 10 mL artificial brackish water, supplied each with 10 mM potassium nitrate and sodium thiosulfate. Prior to adding supplements and inoculation of strain GD1 the medium was bubbled with N_2 and covered with butyl-rubber stoppers. Incubation was done at 15°C in the dark, total incubation time did not exceed seven days. Radioisotope incorporation experiments were done in triplicate, as control served a formol-inactived culture at the same physiological state. To each glass test tube 0.2 µCi sodium-[^{14}C(2)]-pyruvate (specific activity 15 mmol/mCi) was added anaerobically after day 6 and day 4, respectively. After 18 h and 72 h incubation with the radioactive substrate the incorporation was stopped by adding 500 µl 37% formol (sterile-filtered). Aliquots of the culture for DAPI staining were taken before substrate addition and after incubation with pyruvate.

3.3.2 Characterization of label incorporation

All radioactivity measurements were done in a liquid scintillation counter (Perkin Elmer). To determine the total activity of each sample an aliquot of 50 µl was directly measured. From each replicate as well as from the blank 500 µl (triplicate from each sample) was filtered onto a cellulose nitrate filter (0.2 µm pore size) and the radioactivity was determined individually. To estimate the fate of the labeled carbon the remaining culture was filtrated onto polycarbonate filters (0.2 µm pore size). From this filters a nucleic acid extraction modified after Weinbauer et al. (2002) was applied, each obtained fraction was retained for subsequent radioactivity measurements. The filters were shockfrozen on liquid nitrogen and subsequently crunched using micropistills. After adding 500 µl of neutral extraction buffer [2.5% (w/v) sodium lauryl sulfate, 15 mmol sodium acetate L^{-1}, pH 7.5] three

freeze-and thaw cycles with intermittend vortexing followed. To this suspension 500 µl phenol-chloroform (8:1, pH 7.2) was added, vortexed and centrifuged for 5 min at 14.000 g. The aqueous supernatant containing nucleic acids and polar organic compounds were washed once with chloroform. The nucleic acids were subsequently precipitated with 1 volume isopropanol and 0.1 volume of 3 mol potassium acetate L^{-1} and 26 µg Glycogen as a carrier. The pellet was washed once with 200 µl 70% ethanol and after air-drying diluted in 50 µl DEPC-treated water. For radioactive measurements the volume of each fraction did not exceed 500 µl.

3.3.3 Cellular fatty acid and amino acid analyses

The cellular fatty acid analysis is described in detail in (Abraham et al., 1998). Cells from filters were saponified (15% (w/v) NaOH, 30 min, 100° C), methylated to fatty acid methyl esters (FAMEs) (methanolic HCl, 10 min, 80° C) and extracted (hexane/methyl-tert-butyl ether (1:1, v/v)) as described in detail by Osterhout et al. (1991). Amino acid analysis was performed as described elsewhere (Abraham and Hesse, 2003). Briefly, proteins from cells were hydrolyzed using 6M HCl (24h, 100°C). After evaporation and water removal carboxylic groups of the sample were esterified with isopropanol/acetyl chloride (5:1 v/v, 105°C, 45 min). Amino groups of the dried esterified samples were acylated with trifluoroacetic acid anhydride at 100°C for 15 min.

FAMEs and esterified amino acids were analyzed using a Hewlett-Packard (HP) 5890A gas chromatograph. Separation of fatty acid methyl esters was achieved with a fused-silica capillary column (25 m by 0.2 mm) with cross-linked 5% phenyl methyl silicone (film thickness 0.33 µm; HP Ultra 2). The computer-controlled parameters were already described by (Osterhout et al., 1991). The instrument was equipped with a flame ionization detector and an autosampler (HP 7673). H_2 was serving as carrier gas. Separation of amino acid derivatives were performed as described for FAMEs, but the capillary column HP Ultra 2 (50 m by 0.32 mm, film thickness of 0.25 mm) was used. Gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) was performed on a Finnigan MAT 252 isotope ratio mass spectrometer coupled with a HP 5890 gas chromatograph via a combustion interface. The separation and combustion of fatty acids are described in detail in Abraham and Hesse (Abraham and Hesse, 2003). Stable isotope composition was expressed in the δ notation with PeeDee Belemnite as standard (Craig, 1957).

Due to derivatisation of the fatty acids and the amino acids, additional carbon atoms were introduced, which resulted in alteration of the isotope ratios. Therefore, when calculating the carbon isotope ratio, correction was done regarding the isotope ratio of the moieties to

obtain the original isotope ratio of biomarkers using formerly described equations (Goodman and Brenna, 1992; Abrajano Jr. et al., 1994; Silfer et al., 1994; Engel et al., 1995).

3.3.4 Incorporation of ^{13}C-pyruvate by the strain GD1 into nucleic acids

Strain GD1 was grown under anoxic conditions in 400 ml artificial brackish waters, containing vitamins, trace elements, and 10 mmol potassium nitrate and sodium thiosulfate L^{-1} each. Substrate combinations for SIP experiments are given in Table 6.

No.	Bicarbonate	Pyruvate	Growth
1	^{12}C	-	+
2	-	^{13}C(2,3)	-
3	^{13}C	-	+
4	^{13}C	^{12}C	+
5	^{12}C	^{13}C(2,3)	+
6	^{12}C	^{13}C (1)	+

Table 6 Substrate combinations for SIP experiments.

Bicarbonate was added to a final concentration of 2 mM, sodium pyruvate to 80 µM. Growth was determined by DAPI counts. - no growth detectable. + Increase of cell numbers to at least the factor 1000.

After an incubation time of 8 d in the dark at 15°C, 100 ml of the cultures were filtered onto 0.2 µm membrane filters and eventually shock frozen. From these filters RNA was extracted according to Weinbauer et al. (2002) with minor modifications as described in Glaubitz et al. (2009).

Gradient preparation and fractionation was done as previously described (Glaubitz et al., 2009) with minor modifications: Each gradient consisted of 200 ng DNase-treated RNA and precipitated RNA from each fraction was eluted in 100 µl DEPC-treated water. For an estimation of the 16S rRNA copy distribution within the density gradient a qPCR with the primer set OST1F and OST1R (Höfle et al., 2005) specific for the epsilonproteobacterial group GD17 (Table 7) was conducted using the Access one-step-RT-PCR system (Promega) as described before (Glaubitz et al., 2009). Amplification was monitored using 0.1x SybrGreen and 100 µmol fluorescein L^{-1} as reference dye. For an absolute quantification of rRNA molecules, *in vitro* RNA transcripts (approx. 1400 nt) of cloned epsilonproteobacterial nearly full-length 16S rRNA amplicons (Grote et al., 2007) were used in defined dilution steps from 10^8 up to 10^2 copies per qPCR reaction (Lueders et al., 2004a).

Name	Sequence	Target	E. coli Position	Application	Reference
Ba519f	CAG CMG CCG CGG TAA NW	*Bacteria*	519-535	qPCR	(Stubner, 2002)
Ba907r	CCG TCA ATT CMT TTR AGT T	*Bacteria*	907-925	qPCR	(Stubner, 2002)
com1f	CAG CAG CCG CCG TAA TA	*Bacteria*	519-535	SSCP	(Schwieger and Tebbe, 1998)
com2rpH	CCG TCA ATT CCT TTG AGT TT	*Bacteria*	907-926	SSCP	(Schwieger and Tebbe, 1998)
Ost1F	TCA GAT GTG AAA TCC AAT GGC TCA	*GD17 group*	663-686	qPCR	(Höfle et al., 2005)
Ost1R	CTT AGC GTC AGT TAT GTT CCA GG	*GD17 group*	803-825	qPCR	(Höfle et al., 2005)

Table 7 Primer combinations used in this study.

3.3.5 Microscopic determination of cell counts

Cells were filtered onto black Nucleopore Filters (0.2 µm diameter) and subsequently stained with 4',6'-diamidino-2-phenylindol (DAPI). Filters were examined using an epifluorescence microscope (Axioskop 2 mot plus, Zeiss) equipped with a 100x Plan Apochromat oil objective lens (Zeiss) with an appropriate filter set. At least 500 cells in randomly chosen microscopic fields were used for the determination of total cell numbers.

3.3.6 Investigation of bacterial [^{13}C]-pyruvate incorporation in a pelagic redoxcline

Samples were taken onboard of the research vessel „R/V Poseidon" during the cruise P370 in the Landsort Deep (station 284; 58°35'N, 18°35'E) in August 2008 using a conductivity, temperature, and depth probe (Pump CTD) connected with a water sampling device. Physico-chemical profiles of the water column at the sampling location were done as described elsewhere (Grashoff et al., 1983). Dark CO_2 fixation rates were determined onboard as described in Glaubitz et al. (2009) after a modified protocol by Steemann Nielsen (1952).

For SIP experiments water samples taken from 96.5 m referring to the turbidity maximum, sodium-[^{13}C(2,3)]-pyruvate as well as sodium-[^{12}C]-pyruvate were added in a final concentration of 100 µmol L^{-1} to 630 ml redoxcline water. The same concentrations were chosen for the reference substrates sodium-[^{12}C]-acetate and sodium-[^{13}C(1,2)]-acetate. As negative control served unamended incubated water samples as well as directly filtered *in situ* water samples. Incubations were done at *in situ* temperature and in the dark for 24 as well as 72 hours. All incubation experiments were conducted in triplicate. Sacrificed bottles were filtered onto Durapore membrane filters and eventually shockfrozen. The procedure of

nucleic acid extraction, isopycnic centrifugation as well as the subsequent molecular analyses is described in detail in Glaubitz et al. (2009). Applied primers for qPCR and fingerprints analyses are listed in Table 7.

3.4 Results

3.4.1 Uptake of radiolabeled pyruvic acid by GD1

GD1 incorporated 0.0109 fmol [^{14}C]-pyruvate cell^{-1} after 19h and 0.0145 fmol [^{14}C]-pyruvate cell^{-1} after 72 h, respectively (Fig 17), referring to about the half of the added pyruvate.

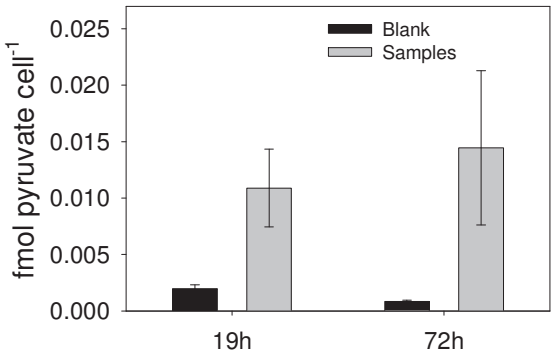

Figure 17 Mean values of cell-specific incorporation of [^{14}C(2)]-pyruvate into GD1 cells.
Error bars indicate standard deviations.

To investigate the destination of the assimilated pyruvate the radioactive signal in different fractions of the cell was analyzed (Fig 18). Due to a signal loss in phenolic fractions of about 40 % the results are expressed in percent of the cumulative signal (sum of signal of all fractions). Approximately 85% of the incorporated signal accumulated in the phenolic fractions, usually containing lipids as well as proteins. In absolute terms the signal accounted for 3.2 to 17.2 amol ^{14}C cell^{-1}. The ethanolic supernatant, containing most probably low molecular, soluble organic compounds, comprised about 13% of the cumulative signal, accounting for 0.7 to 3.1 amol ^{14}C cell^{-1}. In the dissolved nucleic acid pellet only a small portion of radioactivity could be recovered, overall, less than 1% of the cumulative signal was detectable.

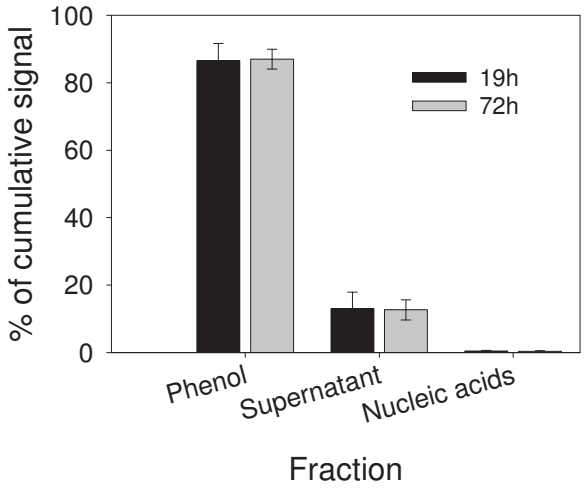

Figure 18 Radioactivity of the different fractions analyzed.

Values are expressed in percent of the cumulative signal, which is defined as sum of all counts of all fractions. Error bars indicate standard deviations.

3.4.2 Incorporation of ^{13}C-pyruvate into fatty acids and amino acids

Mass spectrometric analyses revealed a considerable incorporation of labeled pyruvate into the analyzed amino acids after 8 days incubation time (Fig. 19). Cells grown on [^{12}C]-bicarbonate served as control. The maximal values of control cells accounted for δ^{13}C 71 ‰ (aspartate), in general the values were negative (data not shown).

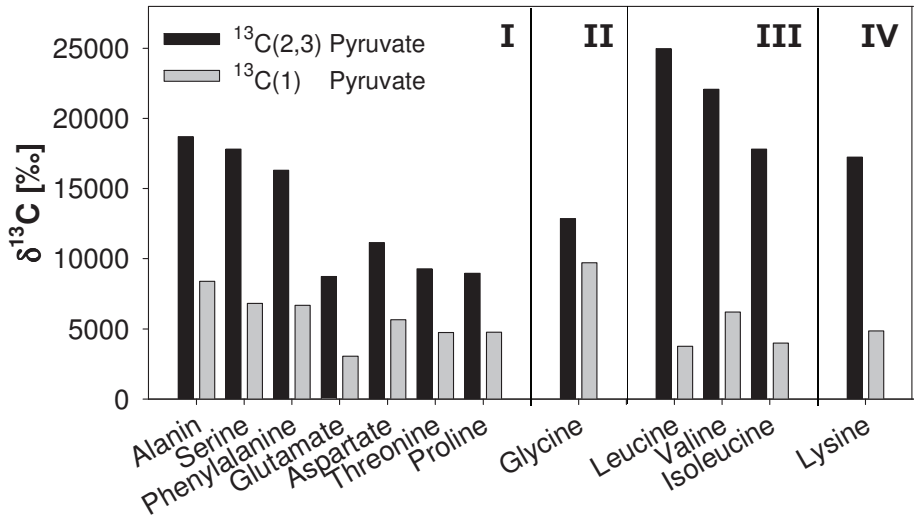

Figure 19 δ^{13}C values of amino acids isolated from a pure culture of GD1.

Only measurements of incubation with [^{13}C]-pyruvate for eight days are shown. The δ^{13}C notation is given in relation to the PDB standard. Roman numerals denote the type of pathway the respective amino acid was presumably synthesized. I: Synthesized from one complete molecule of pyruvate. II: Synthesized from serine, deletion of the C3-position originating from pyruvate. III: Synthesized from condensation of two molecules of pyruvate or derived metabolites.

The maximal $\delta^{13}C$ value was determined for leucine, accounting for 25,000 ‰ after labeling with [^{13}C(2,3)]-pyruvate for 8 days, which equals a ^{13}C content of 29.2 % in this molecule. Using [^{13}C(1)]-pyruvate the $\delta^{13}C$ value in this amino acid accounted for only 3,800 ‰, which corresponds to a ^{13}C content of approximately 5.4 %. Besides leucine, the labeling of valine is comparably high. The lowest labeling was detected in the amino acids glutamate, aspartate, threonine and proline; however, in all investigated amino acids $\delta^{13}C$ values of more than 3,000 ‰ were detectable, accounting for at least 4.5 % total ^{13}C in the respective metabolite.

Three different groups of amino acids, defined by the detected labeling efficiency were distinguishable. The first group was constituted of the amino acids alanine, serine, phenylalanine, glutamate, aspartate, threonine and proline. The amount of incorporated ^{13}C from [^{13}C(1)]-pyruvate labeled samples corresponded approximately for half of the detected label derived from [^{13}C(2,3)]-pyruvate. In the second group, solely represented by glycine, the labeling was nearly equal after incubation with [^{13}C(2,3)]-pyruvate and [^{13}C(1)]-pyruvate. The absolute $\delta^{13}C$ values accounted for 12,000 ‰ ([^{13}C(2,3)]-pyruvate) and 9,700 ‰ ([^{13}C(1)]-pyruvate), accounting for 14.6 and 12.0 % ^{13}C, respectively. The third group was constituted by the amino acids leucine, valine, isoleucine and lysine. The incorporated ^{13}C in the respective amino acid after incubation with [^{13}C(1)]-pyruvate accounted only for 18 - 32% of the determined ^{13}C content compared to the [^{13}C(2,3)]-pyruvate-labeled samples.

Fatty acids of GD1 were labeled to a lesser extent than amino acids (Fig. 20). The maximal $\delta^{13}C$ value of 12,800 ‰ was determined for the main fatty acid C16:1ω7 after incubation with [^{13}C(2,3)]-pyruvate for 8 days, accounting for an absolute ^{13}C content of 15.5 %.

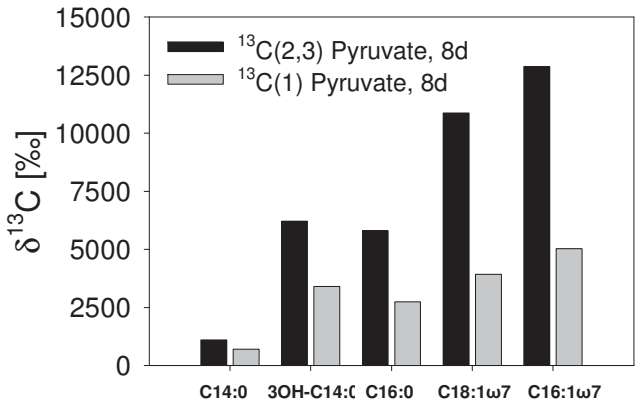

Figure 20 Isotopic composition of selected fatty acid methyl esters (FAME) after incubation with ^{13}C labeled pyruvate for eight days.

Values are expressed in $\delta^{13}C$ notation in relation to the PDB standard.

Incubation with [^{13}C(1)]-pyruvate caused a δ^{13}C value of 5,000 ‰, resulting in an absolute ^{13}C content of 6.7 % ^{13}C in this fatty acid. The comparison between the two different substrates revealed that approximately half of the label was present in [^{13}C(1)]-pyruvate treated samples compared to the [^{13}C(2,3)]-pyruvate labeled samples. Only the fatty acid C14:0 exhibited a different pattern, about 80% of the ^{13}C label was present in the [^{13}C(1)]-pyruvate labeled samples compared to the [^{13}C(2,3)]-pyruvate-treated culture. The δ^{13}C values in this fatty acid were only 1,000 ‰ and 700 ‰ , respectively, accounting for absolute ^{13}C content of 2.3 and 1.9 %.

3.4.3 Pyruvate incorporation into nucleic acids of GD1

The distribution of 16S rRNA copies within the density gradients were analyzed by bacterial qPCR and revealed that an incubation with ^{13}C-pyruvate did not considerably influence the buoyant densities of the rRNA copies (Fig. 21 A,B). More than 95 % of the rRNA copies from cells grown on [^{12}C]-bicarbonate banded between 1.771 and 1.814 g cm^{-3}.

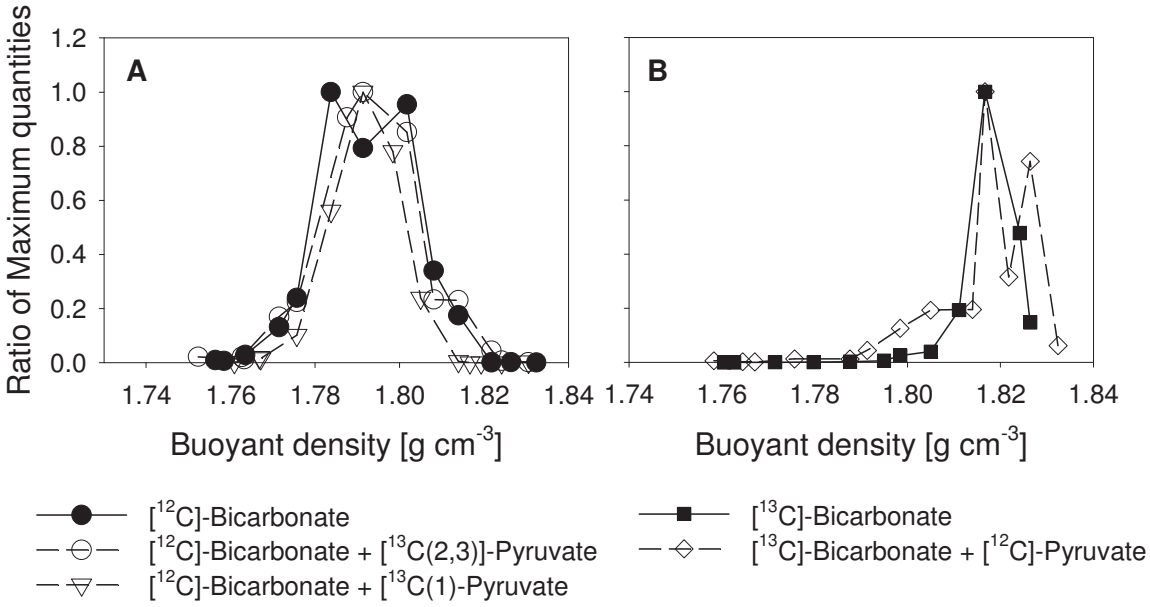

Figure 21 Quantitative distribution of rRNA in CsTFA density gradients prepared from GD1 cells grown eight days with different substrate combinations.

(A) Incubations based on [^{12}C]-bicarbonate. (B) Incubations based on [^{13}C]-bicarbonate. Domain-specific template distribution within gradient fractions was quantified by quantitative reverse transcription-PCR. Data are given as the dimensionless normalization, to allow comparisons between gradients.

The same distribution pattern was detected for incubations with unlabeled bicarbonate and additional [^{13}C]-pyruvate species with copy number maxima at 1.791 g cm^{-1}. The copy number maximum of the [^{12}C]-bicarbonate incubation was detected at 1.784 g cm^{-3}, which was presumably due to a pellet loss or inhibitory residues in resolved RNA (Fig. 21A). rRNA from

cells grown on [^{13}C]-bicarbonate banded between 1.811 and 1.826 g cm^{-3}, the addition of ^{12}C-pyruvate had only minor influence on the buoyant density of the rRNA. This resulted in a broader distribution of rRNA copies within the density range from 1.798 to 1.832 g cm^{-3}, exhibiting a small shoulder between 1.791 to 1.814 g cm^{-3}. Both copy number maxima were detected at 1.817 g cm^{-3} (Fig. 21B).

3.4.4 Pyruvate incorporation in a pelagic redoxcline

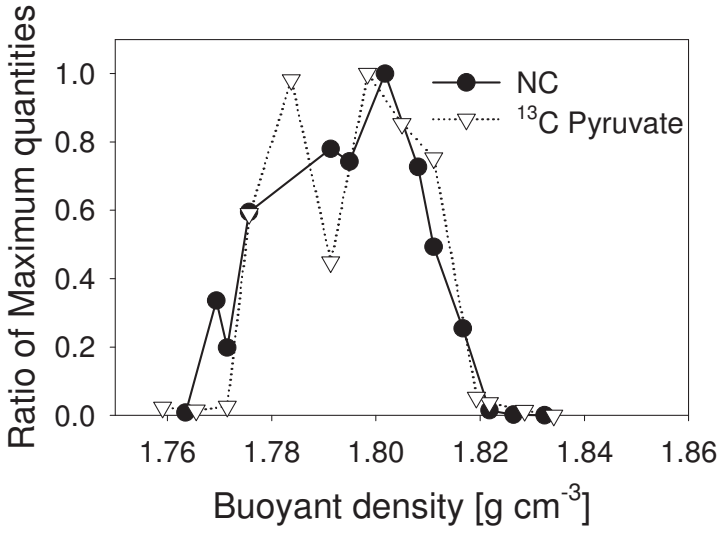

Figure 22 Quantitative distribution of rRNA of a natural community from a pelagic redoxcline in CsTFA density gradients after 72 h of incubation with [^{13}C(2,3]-pyruvate or without additional substrate. Domain-specific template distribution within gradient fractions was quantified by quantitative reverse transcription-PCR. Data are given as the dimensionless normalization, to allow comparisons between gradients.

The analyses of pyruvate uptake were conducted on natural water originating from sulfidic waters of a pelagic redoxcline. The sulfide concentration was 12.73 µM; oxygen was below the detection limit. The CO_2 fixation rate of the natural community accounted for 0.34 µmol L^{-1} d^{-1}. The pyruvate incorporation rate was 0.035 µmol L^{-1} d^{-1}. The estimated turnover, a value expressing the percentage of metabolized substrate per time unit, of the added pyruvate was 0.16 % h^{-1} (data not shown).

rRNA-SIP analyses revealed that incubation with ^{13}C-pyruvate did not affect the buoyant densitity of bacterial 16S rRNA copies (Fig. 22). More than 95% of the transcripts of the negative control and the [^{13}C(2,3)]-pyruvate amended sample were distributed between 1.679 to 1.817 g cm^{-3} and 1.776 and 1.819 g cm^{-3}, respectively. Both copy number maxima were detected at 1.795 g cm^{-3}. SSCP-fingerprints of density resolved 16S rRNA demonstrated a similar distribution of PCR products towards higher densities for all samples investigated (Fig. 23). The GD17 cells were abundant in all treatments, but the maximal relative band

intensity of GD17 was always detected in lighter fractions. SSCP-fingerprinting of RT-PCR products generated from total RNA of different incubation experiments revealed that the addition of organic substrates did not alter the band pattern remarkably (Fig. 24). The only well-separated sample was the negative control after 72 hours of incubation.

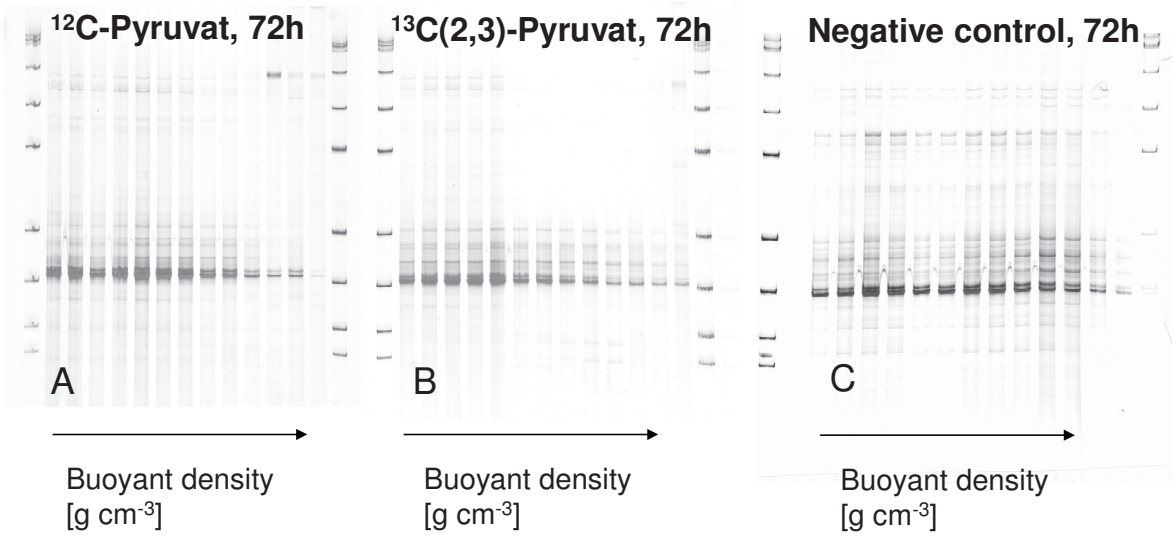

Figure 23 SSCP fingerprints of density-resolved bacterial SSU-RNA templates from CsTFA density gradient fractions of the (A) [^{12}C] - and (B) [^{13}C]-pyruvate incubation experiments.

(C) Negative control without additional substrates. The lanes of each gel represent fractions with increasing buoyant densities (BD), from left to right. Bands from the GD17 cluster are marked.

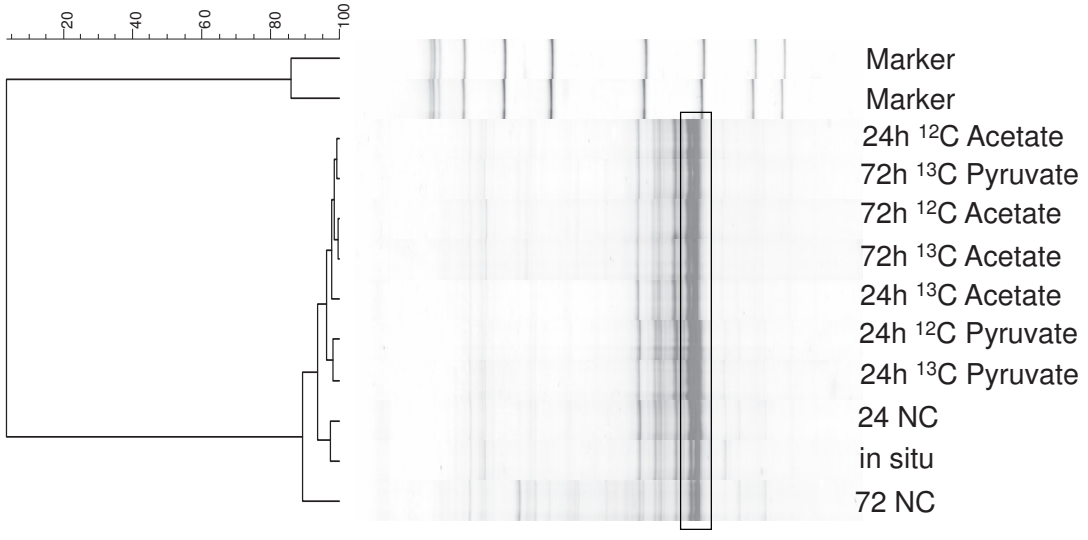

Figure 24 Cluster analyses of RT-PCR products of total RNA from several incubation experiments based on 16S rRNA SSCP fingerprinting.

Boxed area denotes the bands from the GD17 cluster; comparison based on Pearson correlation.

3.5 Discussion

In this study we provided evidence that the obligate chemolithoautotrophic *Sulfurimonas* sp. GD1 is able to use pyruvate as an additional carbon source, with the majority of the assimilated pyruvate used for biosynthesis of amino acids and to a lower extent for fatty acid synthesis. An incorporation of pyruvate in nucleic acids could not be monitored sufficiently by SIP and radiocarbon analyses. Field studies revealed that no pyruvate-assimilating organisms could be identified by RNA-SIP analyses, although an uptake of this carboxylic acid was present as determined by radiocarbon measurements. Our findings compromise the practicability of RNA-SIP analyses for identification of heterotrophic and mixotrophic prokaryotes, but provide useful information of the metabolism of the versatile chemolithoautotroph *Sulfurimonas* sp. GD1.

3.5.1 Fate of pyruvate in the metabolism of GD1

Radiocarbon measurements revealed that ^{14}C-labeled pyruvate was actively incorporated into GD1 cells. Almost 100% of the incorporated signal was recovered in fractions, where lipids, proteins and low-molecular organic compounds are suspected. Neither in radioactive measurements nor in RNA-SIP samples a distinct accumulation of pyruvate-deriving carbon in nucleic acids could be detected. Studies concerning chemoautotrophic *Betaproteobacteria* related to *Thiobacillus* spp. described a similar phenomenon (Taylor et al., 1971). These authors stated that carbon from radioactive acetate and pyruvate was transferred to fatty acids and only four different amino acids. However, the strain GD1 uses the rTCA-cycle for fixation of inorganic carbon (Grote, 2009), which is common among chemoautotrophic *Epsilonproteobacteria* (Takai et al., 2005; Nakagawa et al., 2007; Sievert et al., 2008). In this ancient pathway pyruvate represents a central metabolite and hence, the contribution of pyruvate for nucleic acid synthesis is imaginable to at least a minor part, but the methods applied for analyses the isotopic content of nucleic acids in the present study are potentially not sensitive enough to prove that. Our data suggest that GD1 uses external pyruvate for synthesis of metabolites. The highest isotopic contents were found in amino acids in metabolites, where as little as possible transformations are needed. This was the case for amino acids which were either directly synthesized from pyruvate (i.e. alanine) or by condensation of two molecules pyruvate (i.e. leucine). The effect can be at least partially explained by isotope discrimination in most enzymatic reactions. The isotopic contents as well as the differences in labeling efficiencies due to different substrates allowed us to hypothesize distinct biosynthesis pathways (Fig. 19). The first group was synthesized from metabolites deriving from one complete molecule of pyruvate, which is reflected by the fact that the

amount of incorporated ^{13}C from [$^{13}C(1)$]-pyruvate labeled samples corresponds approximately for a half of the detected label derived from [$^{13}C(2,3)$]-pyruvate. The second group, solely represented by glycine, was synthesized from serine, belonging to group I by transferring the methylen residue from position 3 to tetrahydrofolate by the glycine hydroxymethyltransferase, resulting in a nearly similar isotopic content at both amendments. The percentage of labeled carbon in the respective amino acid in the group III after incubation with [$^{13}C(1)$]-pyruvate may account for just 18 - 32% compared to the labeling with [$^{13}C(2,3)$]-pyruvate. It is reliable that molecules are synthesized from condensation of two molecules of pyruvate with deleting from one pyruvate the carboxylic residue.

Fatty acids are in general synthesized from several molecules malonyl-CoA, depending on chain-length and one molecule of acetyl-CoA. The acetyl-CoA synthesis in the strain GD1 is yet not understood. In aerobic organisms as well as mitochondria acetyl-CoA is synthesized from pyruvate by the pyruvate-dehydrogenase (PDH) complex. Genome analyses of the strain GD1 revealed that this organism lacks two of four subunits of this multienzyme complex. In the pathogenic epsilonproteobacterium *Helicobacter pylori*, that also lacks PDH activity, the synthesis of acetyl-CoA is catalyzed by the pyruvate:ferredoxin oxidoreductase (pyruvate synthase, EC 1.2.7.1) (Hughes et al., 1998). The same enzymatic reaction was reported for several other anaerobic organisms, like *Chlorobium limicola* (Fuchs et al., 1980a) or *Clostridium thermoaceticum* (Furdui and Ragsdale, 2000). In the reversed tricarboxylic acid cycle the carboxylation of acetyl-CoA to pyruvate is catalyzed by this enzyme, thereby reduced ferredoxin is oxidized. Consequently, the reversed reaction to acetyl-CoA leads to reduced ferredoxin, resulting in a gain of energy. The synthesized acetyl-CoA is further carboxylized to malonyl-CoA, which enters the fatty acid synthesis process. Regarding this process, addition of [$^{13}C(1)$]-pyruvate to the GD1 should not lead to an isotopic enrichment in fatty acids, because the C1-position is deleted by decarboxylation during acetyl-CoA synthesis. The fact that we detected an incorporation of the heavy isotope can be explained by different processes. The most probable explanation is the tight coupling (temporal and spatial) of the decarboxylation of pyruvate and the carboxylation of acetyl-CoA, resulting in recycling of the carbon dioxide of the deleted ^{13}C-labeled carboxylic group. Furthermore, by carboxylating the exogenous pyruvate by the pyruvate carboxylase (EC 6.4.1.1), oxaloacetate is formed. This precursor of the amino acid aspartate serves a central metabolite in the rTCA cycle, which can be metabolized further to acetyl-CoA. In this case the C1-position of the pyruvate will be recovered in the C2-position of the acetyl-CoA. This rearrangement of atoms during biosynthesis was already reported in a study concerning the related autotrophic organism

Chlorobium limicola, which also uses the rTCA cycle for inorganic carbon assimilation (Fuchs et al., 1980b).

In our study we detected a relatively low labeling of the fatty acid C14:0 compared to the other investigated fatty acids. Different scenarios are conceivable, but further experiments are needed to disclose the underlying mechanisms. It cannot be excluded that the first steps in fatty acid synthesis mostly have an autotrophic origin. Another explanation is given by the fact that myristic acid is the precursor of all other fatty acids in GD1. Due to constant turnover the labeled pyruvate gets diluted and thus relatively depleted in ^{13}C, whereas the labeled end products accumulate.

Our SIP experiments on the strain GD1 as well as the natural community revealed that only a marginal percentage of pyruvate was used for nucleic acid synthesis. Typical labeling experiments with organic substrates revealed density shifts of the copy number maximum from a partially labeled community of approximately 0.005 g cm^{-3} (Lueders et al., 2004b), 0.007 g cm^{-3} (Egert et al., 2007) and even up to approximately 0.02 g cm^{-3} (Lueders et al., 2004c). Thus, the detected slight labeling effects in this study (Fig. 5B) are considered to be negligible.

3.5.2 Pyruvate usage in a pelagic redoxcline of the central Baltic Sea

Usually the highest dark CO_2 fixation rates in pelagic redoxclines of anoxic basins like the Baltic Sea (Gocke, 1989; Labrenz et al., 2005; Jost et al., 2008), the Black Sea (Jørgensen et al., 1991; Sorokin et al., 1995) or the Cariaco Basin (Tuttle and Jannasch, 1973b; Taylor et al., 2001) are detectable below the chemocline, which is defined as the shallowest appearance of sulfide. The character of the energy-yielding processes for chemosynthesis is still a matter of debate, but the fact that concentrations of potential electron acceptors like oxygen and nitrate are below the detection limit, indicates strong energy depletion in those systems. The ability to use pyruvate or other low-molecular compounds may provide a potentially important ecological niche for obligate chemolithoautotrophs. Due to the energy-consuming character of assimilation of inorganic carbon, the uptake of central precursors for biosynthesis of at least a couple of metabolites may benefit the organism, which was already postulated several decades ago (Rittenberg and Wilkinson, 1969; Kuenen and Veldkamp, 1973). In fact, the additional uptake of acetate was already proven on isolated chemoautotrophs from a Black Sea redoxcline (Jannasch et al., 1991). Recent metagenome and proteome studies on the sulfur-oxidizing symbiont *Candidatus* 'Endoriftia persephone' revealed that this chemolithoautotrophic gammaproteobacterium is at least temporary capable of using

heterotrophic substrates. Accordingly, genes for importers as well as enzymes from the oxidative tricarboxylic acid cycles were found (Markert et al., 2007; Robidart et al., 2008). In this metagenome, but also in the genome of GD1, genes for chemotactical behaviour, which can also be indicative for heterotrophic or mixotrophic uptake of organic compounds, were found. A positive chemotactical reaction towards nitrate was already proven by Grote et al. (Grote, 2009).

At pelagic redoxclines of anoxic basins the existence of potentially heterotrophic *Gammaproteobacteria* belonging to the genus *Pseudoalteromonas* spp. was already proven (Madrid et al., 2001; Vetriani et al., 2003; Labrenz et al., 2007); members of this genus were also shown to be involved in pyruvate uptake in the Arabian Sea (Al-Sarawi et al., 2008). Furthermore, *Arcobacter* spp., potentially involved in manganese reduction, was shown to be stimulated after incubation with low-molecular organic substrates (Beckmann, 2006). The same organism was identified as an acetate-consuming representative by RNA-SIP analyses (Berg, 2010). Thus, the usage of pyruvate and other low-molecular organic compounds should be conceivable in those systems.

In general, concentrations of organic carbon are rather low in aquatic systems. DOC concentrations of 170-280 μM in the Black Sea (Ducklow et al., 2008) and 45-70 μM in the northwestern Sargasso Sea (Goldberg et al., 2009) were determined. The total concentration of DOC at the redoxcline of the Landsort Deep in August 2008 accounted for 280 μM C (K. Nagel, personal communication), whereas the character of the corresponding compounds was not determined further. A study investigating the bioavailability of organic carbon in the Loch Creran, a coastal area in Scotland, revealed that 29% in average of the determined DOC was bioavailable carbon (Lønborg et al., 2009) and thus available for mineralization by heterotrophic microorganisms. This remineralization process is one of the most important components in the microbial loop and in the global carbon budget (Azam et al., 1983).

In our field-experiment we could not observe uptake of pyruvate into nucleic acids of a natural community. To provide evidence for pyruvate-usage on a single-cell level in a natural habitat the employment of MICRO-CARD-FISH protocols should more feasible, because of its independence of the fate of the carbon in the cell carbon cycle. These findings are based on a single experiment from a redoxcline of the central Baltic Sea and need further investigation; however, the experiments on the strain GD1 suggest, that pyruvate was incorporated and metabolized by cells of the GD17 group.

Summary

The aim of this thesis was to provide comprehensive information on the identity and diversity of chemolithoautotrophs in pelagic redoxclines of anoxic marine systems, leading to a better understanding of ecosystem functioning. The knowledge on the mixotrophic potential of the putative key player *Sulfurimonas* sp. GD1 in chemolithoautotrophic denitrification was expanded and first attempts to investigate the heterotrophic community in pelagic redoxclines were done. The culture-independent rRNA-based SIP method combined with molecular techniques was applied, to some extent also in combination with mass spectrometric analyses. This polyphasic approach is advantageous because of the unfocused, non-directional identification of active species, vanquishing the apparently existent disadvantages of culture-dependent methods. Although this method set has its own limitations, the applied approach turned out to be a powerful mean to study chemolithoautotrophic assemblages. For interpretation of the results obtained in the present thesis it has to be taken into consideration that the RNA-based SIP method is a purely qualitative technique, the influence of PCR biases and primer specificity does not allow more than an estimation of quantitative impact of identified prokaryotes. A major outcome of this study is a consequence of the fact that external carbon does not mandatory serve as a compound for nucleic acid synthesis, limiting the RNA-SIP methods.

For the first time a detailed description of chemolithoautotrophic assemblages in mesophilic, sulfidic habitats was provided in this work. The autotrophic production in sulfidic areas of Black Sea and Baltic Sea redoxclines could be exclusively assigned to *Proteobacteria*, excluding *Archaea*. The cumulative results of the present thesis clearly demonstrate the impact of organisms that are most likely involved in sulfur oxidation and denitrification in sulfidic depths of pelagic redoxclines of the central Baltic Sea and the Black Sea. In both systems the impact of *Epsilonproteobacteria* and *Gammaproteobacteria* to dark CO_2 fixation was proven. These phyla are found in numerous anoxic, sulfidic habitats and are increasingly recognized to be key players in element cycling processes. Representatives of the *Epsilonproteobacteria* closely related to the genus *Sulfurimonas*, were found to be abundant in extreme habitats like hydrothermal vents (Polz and Cavanaugh, 1995) (Nakagawa et al., 2005a) and pelagic redoxclines (Lin et al., 2006; Grote et al., 2007; Wakeham et al., 2007). These habitats are usually characterized by high dark CO_2 fixation rates, but a correlation between epsilonproteobacterial cell abundances and the apparent CO_2 fixation maxima could not be demonstrated thus far. Nonetheless, the significance of

this genus for chemolithoautotrophic production in these habitats was recently proven for Baltic and Black Seas redoxclines (Grote et al., 2008). The authors demonstrated that the chemolithoautotrophic *Epsilonproteobacteria* in the Baltic Sea are principally constituted of one single cluster, closely related to *Sulfurimonas denitrificans* (Grote et al., 2007, 2008); for the Black Sea comparable statements on the composition of the chemolithoautotrophic epsilonproteobacterial community did not exist until now. In the present thesis these finding on the diversity of epsilonproteobacterial chemolithoautotrophs in a Baltic Sea redoxcline were confirmed; moreover, evidence for a potentially reduced diversity of autotrophic *Epsilonproteobacteria* in Black Sea redoxclines was provided. These results lead to the hypothesis that a low diversity is a general feature in pelagic redoxclines, reflecting a presumably perfect adaptation and pronounced metabolic versatility of this taxonomical group. This hypothesis can be supported by earlier diversity studies of anoxic basins, which demonstrated that the diversity of *Epsilonproteobacteria*, related to *Sulfurimonas* spp. was indeed reduced (Madrid et al., 2001; Vetriani et al., 2003). It becomes apparent that the anoxic basins, though geographically well separated, are in principal comparable and analogical. This is also true for important biogeochemical cycles relevant in these systems, e.g. sulfur and nitrogen cycles.

The Gammaproteobacteria detected in this thesis, belong to the so-called GSO cluster, taxonomically yet unclassified. In both anoxic basins the chemolithoautotrophic potential was demonstrated, but the quantitative impact of this group is presumably rather low. The abundance of total *Gammaproteobacteria* was recently determined for redoxclines of the Black Sea and the Cariaco Basin (Lin et al., 2006); in both systems maximal cell counts of approximately 6% of total DAPI counts were detected. For both basins the probe GAM42a (Manz et al., 1992) was employed, which targets the 23S rRNA and whose specificity was recently questioned (Siyambalapitiya and Blackall, 2005). However, in the last years the impact of this taxonomical group was recognized as significant, especially in carbon and sulfur cycling. Symbiotic GSO representatives are of major importance in dark CO_2 fixation, and hence being a substantial basis for ecosystems in deep sea habitats (Ruby et al., 1981; Wirsen et al., 1986; Fisher et al., 1989; Moyer et al., 1995). Moreover, in the last years the significance of this group in sulfur cycling processes was proven. In the OMZ at the African shelf in the South Atlantic cells belonging to the GSO cluster were found to be responsible for the withdrawal of toxic sulfide, establishing an anoxic, but non-sulfidic buffer zone (Lavik et al., 2009). The occurrence of these *Gammaproteobacteria* in numerous sulfidic habitats (Vetriani et al., 2003; Labrenz et al., 2007; Stevens and Ulloa, 2008;

Schmidtova et al., 2009; Walsh et al., 2009) may lead to the conclusion that especially this group may fulfill a key function in sulfur transformation processes. Lavik et al. (2009) hypothesized that blooms of GSO and other sulfide-oxidizing prokaryotes could even disguise massive sulfide appearances in numerous oxygen-depleted habitats worldwide and hence preventing the ecosystems from mass mortality of metazoans. In the present thesis, the contribution of sulfur oxidizing *Gammaproteobacteria* to chemolithoautotrophy was proven in both investigated pelagic redoxclines. In the Baltic Sea redoxcline the maximal activity of these organisms was detected after a short incubation time of 24 hours, whereas after 72 hours the activity of these representatives was strongly reduced. In the Black Sea redoxcline a similar phenomenon was observed; though a pronounced diversity of the GSO cells it was noticeable that after 72 hours incubation only few representatives were actively fixing CO_2. These results strongly suggest that putative electron acceptors, presumably oxygen or nitrate were limiting in these incubations. Interestingly, a close relative to a representative of the GSO, which was found to be chemolithoautotrophic in a Baltic Sea redoxcline, was highly abundant, but not active in the Black Sea redoxcline. This may corroborate the hypothesis of the lack of electron acceptors, but can also point to different carbon acquirement strategies, i.e. non-autotrophic growth. Grote et al. (2008) determined for the Black Sea an epsilonproteobacterial contribution to CO_2 fixation of 70% - 100%. Interestingly, the employed oligonucleotide probe EPS914 (Loy et al., 2007) seems to be less stringent as previously assumed. A nearly identical target sequence was detected for representatives of the GSO cluster of the Black Sea (Table 8), with only one and two mismatches within the target sequences on the same *E.coli* position, respectively.

Organism	Target sequence
BS-GSO1	ACU CAA AGG AAU **U**GA CGG GGA CCC
BS-GSO2	ACU CAA AGG AAU **U**GA CGG GG**G** CCC
Sulfurimonas denitrificans	ACU CAA AGG AAU AGA CGG GGA CCC

Table 8 Target sequence of the oligonucleotide probe EPS914 (Loy et al., 2007).

Mismatches are indicated by bold and underlined letters. All targets occupy the E.coli position 914.

Albeit the contribution of other taxonomical groups to autotrophic processes was hypothesized for sulfidic areas of pelagic redoxclines, no more than *Gamma-* and *Epsilonproteobacteria* were detected in the present thesis. This may be a consequence of the incubation-dependent SIP approach; underrepresented organisms or special requisitions of

nutrients can prevent a successful identification. Spiking with limiting nutrients or incubations under low-light conditions could be a remedy to detect further organisms, possibly involved in autotrophic processes at pelagic redoxclines.

The energy-yielding pathways for chemoautotrophic production are still a matter of debate, recent studies suggest at least for *Epsilonproteobacteria* related to *Sulfurimonas denitrificans* the inorganic disproportionation of thiosulfate (Grote, 2009; Jost et al., 2010). This point was not addressed further in this thesis, but the results of our incubation-dependent analyses suggest, that the driving element was at least partially not limiting for *Epsilonproteobacteria*. Either potential electron acceptors were introduced during bicarbonate-addition or alternative electron acceptors, e.g. metals or thiosulfate fueled the autotrophic production.

The third chapter of this thesis was dedicated to the metabolic and mixotrophic potential of the strain GD1 and a natural chemoautotrophy-dominated prokaryotic community from pelagic redoxclines in the central Baltic Sea. The laboratory experiments provided evidence for a mixotrophic potential of this key organism. At least under denitrifying, chemoautotrophic conditions pyruvate, a potential key metabolite in the metabolism of GD1 was incorporated and served as an additional carbon source. These experiments also revealed that carbon from pyruvate did not contribute to nucleic acids synthesis. The same phenomenon was described in numerous studies dealing with organic nutrition of chemolithoautotrophs (Taylor et al., 1971; Matin, 1978). Thus, the application of RNA-SIP is probably not the appropriate mean to detect mixotrophic prokaryotes. In theory, to detect heterotrophic prokaryotes this substrate and the RNA-SIP technique should be applicable. The use of low-molecular organic compounds by chemolithoautotrophs, no matter if obligate or facultative, can provide an important ecological niche for the respective organism. The provision of intermediate metabolites may be a crucial mean to overcome energy limitations in oligotrophic, energy-depleted habitats like pelagic redoxclines. Moreover, the existence of putative heterotrophic representatives was already proven in sulfidic water layers (Labrenz et al., 2007), and incubation with a mix of organic compounds was successful in stimulating organisms (Beckmann, 2006). Our field study did not succeed in identification of heterotrophic and mixotrophic bacteria, not even a stimulation of a distinct organism without changes in buoyant density was detectable. Nonetheless, the experiments on the cultivated GD1 provided insight into its carbon metabolism. As demonstrated earlier, this strain has the

potential of mixotrophic growth, with pyruvate serving as a basis for synthesis of amino acids and fatty acids.

Future prospects

The results of this thesis provided useful insights in the composition of chemolithoautotrophic assemblages in pelagic redoxclines of anoxic basins, leading to a better understanding of those habitats. The contribution of *Gammaproteobacteria* and *Epsilonproteobacteria* to chemolithoautotrophic production was proven, leading to the hypothesis that these organisms may serve as a basis for a secondary microbial food web.
Until now, nothing is known about the abundance of putative chemolithoautotrophic *Gammaproteobacteria* within the GSO cluster in pelagic redoxclines of the central Baltic Sea or the Black Sea. The abundance of the phylum *Gammaproteobacteria* in the Black Sea and the Cariaco Basin was already investigated (Lin et al., 2006), but the universal probe GAM42a was employed, targeting the 23S rRNA (Manz et al., 1992). As the 23S rRNA of the chemolithoautotrophic GSO representatives identified in this study is not known so far, the applicability of the oligonucleotide probe GAM42a cannot currently be evaluated. Hence, the application of a newly designed oligonucleotide probe targeting the 16S rRNA of the GSO will be an important step towards the understanding of the ecological role of this group. The SIP analyses of the Black Sea community revealed that presumably just a part of the detected representatives of the GSO were actively fixing CO_2, albeit this analysis has to be considered as a purely qualitative approach. The application of a MICRO-CARD-FISH protocol should be successful in quantification the total abundance of GSO cells and estimation of quantitative impact of this group to the total chemolithoautotrophic production. Furthermore, a potential overestimation of the epsilonproteobacterial abundance can be relativized.

An approach to delve the ecological significance of putative chemolithoautotrophs is to investigate the diversity of functional genes involved in element cycling. For the Baltic Sea this has been already done for denitrification genes (Hannig et al., 2006; Falk et al., 2007) and CO_2 fixation genes (Kießlich, 2008), but no detailed information on genes from sulfur-transformation processes exist hitherto. The diversity of these genes is of major importance, since the sulfur transformation processes are considered to be the major energy-yielding process in this habitat. Adequate genes involved in sulfur oxidation processes are the dissimilatory adenosine 5'-phosphosulfate reductase (*AprBA*) and the reverse dissimilatory sulfite reductase (*rDsrAB*), which are key genes in sulfur-oxidizing

prokaryotes. It has to be taken in consideration, that at least the *AprBA* gene is also characteristic for sulfate-reducing prokayotes. These genes were already investigated in oceanic minimum zones (Lavik et al., 2009), alkaline lake sediments (Loy et al., 2009) and sediments of the Caribbean (Meyer and Kuever, 2007b). For the *rDsrAB* gene it has been shown that phylogenetic trees based on the obtained sequence information of are congruent with phylogenetic trees based on 16S rRNA of the accordant sulfur-oxidizing prokaryotes (Meyer and Kuever, 2007a; Loy et al., 2009), contradicting a putative horizontal gene transfer. This approach will provide deeper insights into the sulfur-oxidizing community by identifying yet unknown prokaryotes, not mandatory involved in CO_2 fixation.

Another point which should be addressed in more detail is the contribution of potentially chemolithoautotrophic *Crenarchaeota* to dark CO_2 fixation. Recently it has been shown that representatives of the Marine Group I within the *Crenarchaeota* are highly abundant in the suboxic zone of pelagic redoxclines of the central Baltic Sea (Labrenz et al., 2010). It is well known by now that *Candidatus* "Nitrosopumilus maritimus" and other representatives of this phylum are actively fixing CO_2 (Könneke et al., 2005; Hallam et al., 2006; Ingalls et al., 2006), but for the Baltic such activities have never been shown. Thus, the role of chemolithoautotrophic *Crenarchaeota* in Baltic Sea redoxclines should be confirmed. Unfortunately, CO_2 fixation rates apparent in the suboxic layers in pelagic redoxclines are generally low (Jørgensen et al., 1991; Taylor et al., 2001; Jost et al., 2008), therefore RNA-SIP analyses will be very challenging and possibly not successful. MICRO-CARD-FISH analyses or mass-spectrometric analyses of *Crenarchaeota*-specific biomarkers would be more promising (Wuchter et al., 2003). Moreover, the detection and quantification of transcripts of genes potentially involved in CO_2 fixation will provide further evidence for the contribution of these prokaryotes in chemolithoautotrophic production.

In the present thesis a transfer of the ^{13}C-label from bicarbonate to the ciliate *Euplotes* sp. was observed, which deserves further attention. The employed methods were not sufficient to decide whether it was a consequence of a symbiotic relationship or to grazing of chemolithoautotrophic bacteria. It is well known that ciliates can harbor bacterial symbionts like *Polynucleobacter* spp.. Albeit an ectosymbiont of the huge ciliate *Zoothamnium niveum*, which is embedded in the GSO cluster was identified (Rinke et al., 2006, 2009), a direct evidence for chemolithoautotrophic growth of the symbionts does not exist so far. To delve the relationship between these microorganisms two principal

approaches are applicable. To investigate potential grazing effects experiments with labeled bacteria should be applied. Recently a bait-prey relationship of ciliates and bacteria was demonstrated by applying an RNA-SIP protocol with ^{13}C-labeled bacteria (Moreno et al., 2010). A potential symbiosis should be investigated by FISH-analyses. This will be very challenging because the fragile ciliates are supposed to be lysed during the standard CARD-FISH procedure. Thus, a modified sampling and hybridization protocol, already described for a symbiosis between ciliates and methanogenic bacteria (Shinzato et al., 2007) should be applied to test this hypothesis.

Despite the advances in molecular microbiology the isolation or at least enrichment of putative key players from natural habitats is essential. This has been done successfully for a member of the epsilonproteobacterial subgroup GD17 from a pelagic redoxcline of the central Baltic Sea (Grote, 2009). The autecological studies as well as the genome analyses of this potential key player have provided useful insights into the physiology and ecology of the GD17 group and may lead to a comprehensive view on processes on sulfidic habitats. To my knowledge, hitherto no isolates of representatives of the GSO group exist. This may be based on the dependence of symbiotic bacteria on their host, whereas recent studies revealed that these organisms may appear free-living (Gros et al., 2003; Harmer et al., 2008). Nonetheless, the establishment of a pure or enrichment culture of free-living GSO cells from pelagic redoxclines will be important for further characterization of this special habitat and its microbial community.

A question which could not be answered in this thesis was the usage of pyruvate by a natural community. Though SIP analyses were conducted successfully for acetate in a pelagic redoxcline in the central Baltic Sea (Berg, 2010), no pyruvate-using prokaryotes were identified in the present thesis. ^{14}C-labeled pyruvate was incorporated, but RNA-SIP analyses did not reveal a successful identification. A first step towards the identification of pyruvate-using microorganisms will be the analysis of fatty acids profiles of [^{13}C]-pyruvate augmented water samples originating from a pelagic redoxcline. Laboratory studies on the isolated strain *Sulfurimonas* sp. GD1 revealed that this substrate contributes to a significant part to fatty acid synthesis, and thus an alteration of isotopic composition in fatty acids from a natural community is expected. Furthermore, the application of a suitable MICRO-CARD-FISH protocol should succeed in identification of pyruvate-using microbes. Due to its independence of the fate of carbon in the respective organism even marginal uptake ratios will lead to a sufficient signal. Both methods suffer from low taxonomical resolution,

but will anyhow lead to a better understanding of the microbial community of pelagic redoxclines.

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List of Abbreviations

AMV	Avian myeloblastosis virus
ANOSIM	Analysis of similarities
AOB	Ammonia oxidizing bacteria
AprBA	Dissimilatory adenosine 5'-phosphosulfate reductase
ATP	Adenosine triphosphate
BD	Buoyant density
bp	Base pair
BLAST	Basic local alignment search tool
CARD-FISH	Catalyzed reporter deposition fluorescence in situ hybridization
cDNA	complementary DNA
Ci	Curie
CoA	Coenzyme A
CsTFA	Cesium trifluoroacetate
CTD	conductivity, temperature, depth
DAPI	4',6'-Diamidino-2-phenylindol
DEPC	Diethyl pyrocarbonate (synonym: diethyl dicarbonate)
DGGE	Denaturing gradient gel electrophoresis
DFG	Deutsche Forschungsgemeinschaft
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DOM	Dissolved organic carbon
EC	Enzyme commission
E.coli	Escherischia coli
EDTA	Ethylendiamine tetraacetic acid
FAME	Fatty acid methyl ester
Fig	Figure
GC-C-IRMS	Gas chromatography-combustion-isotope ratio mass spectrometry
GSO	Gamma sulfur oxidizer
MCI	Marine cluster I

MICRO-CARD-FISH	Catalyzed reporter deposition fluorescence in situ hybridization combined with microautoradiography
NADP	Nicotinamide adenine dinucleotide phosphate
nt	Nucleotide
OTU	Operational taxonomical unit
PCR	Polymerase chain reaction
PDB	PeeDee Belemnite
qPCR	Quantitative polymerase chain reaction
rDsrAB	Reverse dissimilatory sulfite reductase
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
rTCA	Reverse (reductive) tricarboxylic acid cycle
RT-PCR	Reverse transcription polymerase chain reaction
R/V	Research Vessel
SDS	Sodium dodecyl sulfate
SIP	Stable isotope probing
SOB	Sulfur oxidizing Bacteria
SSCP	Single strand conformation polymorphism
SRB	Sulfate reducing bacteria
Tris	Tris(hydroxymethyl)aminomethane
U	enzyme unit
V	Volume

Appendix I

Summary of all sequenced clones in all clone libraries generated in the second chapter. * markes the clones, which are included in the phylogenetic tree. [1] defines clones of the GSO cluster BS-GSO1, [2] defines clones from the cluster BS-GSO2. Closest relatives were determined by NCBI-BLAST against the nucleotide collection database. n.a. not applicable.

OTU	Relative abundance of clones [%]				Accession number	Closest relative (accession number, % identity)
	^{12}C-A	^{12}C-B	^{13}C-A	^{13}C-B		
Gammaproteobacteria						
GSO						
1	1.3	0.7	3.6	n.a.	GU108526	Thiotrophic endosymbiont of *Idas* sp. (AM402957; 95%)
3*	3.5	3.7	4.5	1.0	GU108545	*Thyasira flexuosa gill* symbiont (L01575; 97%)
6*[1]	26.7	36.3	16.5	38.8	GU108557	Thiotrophic endosymbiont of *Idas* sp. (AM402957; 98%)
7*[1]	16.1	26.7	13.0	29.1	GU108558	Thiotrophic endosymbiont of *Idas* sp. (AM402957; 98%)
16	2.25	0.74	3.57	n.a.	GU108531	Thiotrophic endosymbiont of *Idas* sp. (AM402957; 94%)
18*	1.61	1.48	6.25	n.a.	GU108533	Endosymbiont of *Lucinoma* sp. (FM213432; 90%)
19*[2]	1.93	2.22	3.57	10.68	GU108534	Endosymbiont of *Ifremeria nautilei* (AB238959; 90%)
20*[2]	3.22	0.74	5.80	5.83	GU108536	Endosymbiont of *Ifremeria nautilei* (AB238959; 90%)
21*	0.32	n.a.	n.a.	n.a.	GU108537	Thiotrophic endosymbiont of *Idas* sp. (AM402957; 93%)
24*[1]	0.32	0.74	0.45	n.a.	GU108540	Thiotrophic endosymbiont of *Idas* sp. (AM402957; 98%)
29*	0,64	n.a.	0,45	n.a.	GU108564	Endosymbiont of *Bathymodiolus heckerae* (AM931532; 93%)
44*	n.a.	n.a.	0.45	n.a.	GU108566	*Thyasira flexuosa* gill symbiont (L01575; 95%)

47*	n.a.	n.a.	0.45	n.a.	GU108568	Thiotrophic endosymbiont of *Idas* sp. (AM402957; 94%)
49*	n.a.	n.a.	0.45	n.a.	GU108570	Endosymbiont of *Bathymodiolus heckerae* (AM236328; 94%)
Altermonadales						
2	4.2	1.5	1.3	n.a.	GU108535	*Colwellia psychrerythraea* (CP000083; 97%)
4	4.8	3.0	1.3	n.a.	GU108554	*Pseudoalteromonas* sp. (AF022407; 95%)
5	4.8	n.a.	2.7	1.9	GU108556	*Pseudoalteromonas* sp. (FJ404721; 99%)
8	0.96	2.22	n.a.	0.97	GU108559	*Colwellia* sp. (AB373115; 96%)
9	4.82	5.19	1.79	n.a.	GU108560	*Pseudoalteromonas* sp. (FJ461431; 95%)
10	0.96	n.a.	0.45	n.a.	GU108561	*Alteromonas* sp. (EU016171; 94%)
12	3.22	1.48	1.34	0.97	GU108528	*Alteromonas* sp. (FJ404749; 95%)
13	0.96	2.22	3.57	n.a.	GU108529	*Marinobacter hydrocarbonoclasticus* (DQ768638.1; 99%)
14	2.25	1.48	3.13	0.97	GU108530	*Pseudoalteromonas* sp. (FJ404721; 99%)
22	0.32	n.a.	n.a.	n.a.	GU108538	*Alteromonas* sp. (FJ404749; 98%)
26	0.32	0.74	1.34	n.a.	GU108542	*Pseudoalteromonas citrea* (AF529062; 92%)
28	0.96	0.74	0.45	n.a.	GU108544	*Colwellia psychrerythraea* (CP000083; 97%)
34	1.29	n.a.	0.45	n.a.	GU108549	*Alteromonas* sp. (FJ404749; 97%)
37	1.29	n.a.	1.79	1.94	GU108551	*Pseudoalteromonas citrea* (AF529062; 96%)
38	0.32	n.a.	0.89	n.a.	GU108552	*Colwellia piezophila* (NR_024805; 90%)
39	0.64	n.a.	0.45	n.a.	GU108553	*Pseudoalteromonas* sp. (AY669166; 95%)
41	0.64	1.48	1.79	n.a.	GU108555	*Colwellia* sp. (FJ952826; 91%)
52	n.a.	n.a.	0.45	n.a.	GU108572	*Alteromonas genoviensis* (FJ040187; 90%)
Oceanospirillales						
27	0.32	n.a.	0.89	n.a.	GU108543	*Alcanivorax* sp. (GQ169073; 93%)

30	0.64	n.a.	2.23	n.a.	GU108546	*Alcanivorax venustensis* (EU440996; 99%)
33	1.61	n.a.	1.34	n.a.	GU108548	*Halomonas* sp. (DQ270715; 92%)
46	0.96	2.22	2.23	0.97	GU108567	*Alcanivorax venustensis* (EU440996; 97%)
Chromatiales						
11	1.29	1.48	4.46	2.91	GU108527	*Rheinheimera* sp. (AM110966; 96%)
17	0.96	0.74	0.45	n.a.	GU108532	*Rheinheimera baltica* (AJ441082; 96%)
25	0.64	n.a.	n.a.	n.a.	GU108541	*Rheinheimera* sp. (FJ527418; 91%)
51	n.a.	n.a.	0.45	n.a.	GU108571	*Rheinheimera baltica* (AJ441082; 94%)
Pseudomonadales						
35	0.32	0.74	1.79	n.a.	GU108565	*Pseudomonas pachastrellae* (EU603457; 93%)
Methylococcales						
48	n.a.	n.a.	0.89	n.a.	GU108569	*Methylobacter tundripaludum* (AJ414655; 98%)
Alphaproteobacteria						
15	0.32	0.74	0.45	n.a.	GU108563	*Paracoccus homiensis* (DQ342239; 99%)
23	0.64	n.a.	n.a.	n.a.	GU108539	Uncultured alpha proteobacterium (AF353236; 96%)
Planctomyces						
36	0.32	0.74	0.45	1.94	n.a.	*Planctomyces maris* (NR_025327; 87%)

Curriculum vitae

School

09/1992 - 06/1998	Secondary school: Otto-Brenner-Gymnasium in Frankfurt/Oder, Abitur

Studies

10/1998 - 08/2004	Studies of biology at the University of Rostock Exams in plant physiology, biochemistry, molecular biology, and biotechnology Diploma Thesis: "Untersuchungen des Folatstoffwechsels in MRP-Transportermutanten in *Arabidopsis thaliana*"
09/2004 - 08/2005	Research assistant, University of Rostock (Prof. Dr. Herrmann Bauwe)
01/2006 - 10/2006	Research assistant, Leibniz-Institute for farm animal biology (PD Dr. habil. Monika Schweigel)
11/2006 - 10/2009	PhD student, Leibniz Institute for Baltic Sea Research Warnemünde (PD Dr. habil. Matthias Labrenz, Prof. Dr. Klaus Jürgens)

Publications and conferences

Publications

Contents of Chapter I have already been published. The content of Chapter II has been accepted for publication. Chapter III is to be submitted in near future. The contribution of the authors to the manuscripts in indicated in the following.

Chapter I (published in peer-reviewed journal)

Glaubitz, S., Lueders, T., Abraham, W.-R., Jost, G., Jürgens, K., and Labrenz, M. (2009) ^{13}C-isotope analyses reveal that chemolithoautotrophic *Gamma-* and *Epsilonproteobacteria* feed a microbial food web in a pelagic redoxcline of the central Baltic Sea. *Environmental Microbiology* **11**: 326-337.

Practical work: S.G. (density gradient centrifugation, qPCR, fingerprints) T.L. (density gradient centrifugation), W.-R.A. (mass spectrometric analyses) G.J. (sampling, chemical parameters, CO_2 fixation rates, ^{13}C-bicarbonate incubations), M.L. (sampling, ^{13}C-bicarbonate incubations, phylogenetic analyses).

Concept and first edition of manuscript: S.G., revision of manuscript: T.L., W.-R.A., G.J.,K.J.,M.L.

Chapter II (accepted for publication in a peer-reviewed journal)

Glaubitz S., Labrenz M., Jost G., and Jürgens K. (2010) Diversity of active chemolithoautotrophic prokaryotes in the sulfidic zone of a Black Sea pelagic redoxcline as determined by rRNA-based stable isotope probing. *FEMS Microbiology Ecology* (accepted)

Practical work: S.G. (density gradient centrifugation, qPCR, fingerprints, cloning, phylogenetic analyses), G.J. (sampling, CO_2 fixation rates, ^{13}C bicarbonate incubations), K.J. (sampling, ^{13}C bicarbonate incubations).

Concept and first edition of manuscript: S.G., revision of manuscript M.L., G.J., K.J.

Chapter III (in preparation)

Glaubitz S., Abraham W.-R., Jost G., Jürgens K., and M. Labrenz. On the role of pyruvate as carbon source in a chemoautotrophy-dominated microbial community in a pelagic redoxcline of the central Baltic Sea.

Practical work: S.G. (cultivation GD1, ^{14}C pyruvate incorporation studies, ^{13}C pyruvate incorporation studies, rRNA-SIP of GD1 and natural samples) W.-R.A. (mass spectrometric analyses), J.G. (sampling onboard, ^{14}C pyruvate incorporation studies)
Concept and first edition of manuscript: S.G., revision of manuscript M.L., K.J.

Conferences

Glaubitz S., Klaus Jürgens, Günter Jost, Tillmann Lueders, Matthias Labrenz **Impact of *Sulfurimonas* – related bacteria on the microbial food web in pelagic redoxclines of the Baltic and Black Sea as determined by ^{13}C analyses** VAAM Tagung Bochum, 8.-11. 03. 2009. (Poster)

Glaubitz S., Lueders T., Abraham W.-R., Jost G., Jürgens K., Labrenz M. (2009) **Chemolithoautotrophic *Proteobacteria* feed a microbial food web in a pelagic redoxcline of the central Baltic Sea as determined by ^{13}C analyses** ASLO Aquatic Sciences Meeting 2009, Nizza, Frankreich, 25.01.-30.01. (Poster)

Glaubitz S., Lueders T., Abraham W.-R., Jost G., Jürgens K., Labrenz M. (2008) **Identification and localization of chemolithoautotrophic organisms in pelagic redoxclines of the central Baltic Sea as determined by ^{13}C isotope analyses** VAAM-Tagung Frankfurt (Main), 9.-11.03. 2008 (Poster)

Kießlich K., Glaubitz S., Jost, Jürgens K., Labrenz M. (2008) **Diversity of two different carbon dioxide fixation pathways in pelagic redoxclines of the Baltic Sea** VAAM-Tagung Frankfurt (Main), 9.-11.03. 2008 (Poster)

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Erklärung

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, den 30.06.2010

Sabine Glaubitz