

## High abundance and dark CO<sub>2</sub> fixation of chemolithoautotrophic prokaryotes in anoxic waters of the Baltic Sea

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### *Abstract*

We determined the abundance and distribution of chemolithoautotrophic prokaryotes in the redoxcline in two basins (Gotland Deep, Landsort Deep) of the central Baltic Sea by combining dark CO<sub>2</sub> fixation measurements with flow cytometric cell sorting. Maximum CO<sub>2</sub> fixation rates were recorded in sulfidic waters about 20 m below the chemocline. Flow cytometric analyses of deoxyribonucleic acid (DNA)-stained bacterioplankton revealed the existence of at least five different prokaryotic clusters in water samples collected below the chemocline. Dark CO<sub>2</sub> fixation in these clusters was determined by flow cytometric sorting after anoxic incubations with NaH<sup>14</sup>CO<sub>3</sub> tracer. Two clusters, representing about 30% of total prokaryotes, were responsible for 65% to 100% of the total dark fixation. Calculated cell-specific CO<sub>2</sub> fixation rates in the two basins ranged from 3.5 to 24.7 fg C cell<sup>-1</sup> d<sup>-1</sup> and suggested that these clusters are dominated by chemolithoautotrophic prokaryotes. Mean cell-specific fixation rates reached more than 10 fg C cell<sup>-1</sup> d<sup>-1</sup> in most cases, indicating relatively high growth rates (doubling times 1–2 d) of chemolithoautotrophic prokaryotes. Our results provide the first evidence of such high cell-specific CO<sub>2</sub> uptake and abundance of chemolithoautotrophic prokaryotes in a pelagic marine environment. However, the identity of the organisms as well as the mechanisms fueling CO<sub>2</sub> dark fixation in the anoxic zone remain unknown.

Chemolithotrophic prokaryotes gain energy by oxidizing inorganic reduced compounds, such as ammonia or hydrogen sulfide, which originate from the degradation of organic material. This prokaryotic activity greatly impacts biogeochemical cycles, especially nitrogen and sulfur cycles in oligotrophic systems. In these environments, chemolithoautotrophs benefit from their ability to grow by assimilating carbon dioxide (CO<sub>2</sub>). The oxic–anoxic interface is an ideal biotope for organisms such as nitrifying or sulfur-oxidizing prokaryotes (e.g., Paerl and Pinckney 1996; Nealson 1997). However, due to the potentially low energy yield of several chemolithotrophic reactions, the

compounds necessary for the reduction of CO<sub>2</sub> are present, but the biomass and growth rates of these prokaryotes are often relatively low (Shively et al. 1998; Kelly 1999; Kelly and Wood 2000).

The Baltic Sea is one of the world's largest brackish-water environments, and it is characterized by a permanent halocline at 60–80-m depth. The oxygen supply of the deep water below the halocline depends mainly on saltwater inflows from the North Sea (Rahm 1987). Pelagic redoxclines, here defined as transition zones of several-meter thickness between suboxic and sulfidic water layers, have become nearly permanently present in the deep basins of the central Baltic Sea because of the decreasing frequency of major saltwater inflows since 1977 (Pers and Rahm 2000). Dark CO<sub>2</sub> fixation at the redoxcline can reach about 30% of surface primary production (Detmer et al. 1993). High rates of chemolithoautotrophic production, measured as dark CO<sub>2</sub> fixation, have also been reported from other marine redoxclines, such as the Black Sea (Jørgensen et al. 1991) and the Cariaco Trench (Taylor et al. 2001). Interestingly, the observed dark CO<sub>2</sub> fixation cannot solely be accounted for by flux calculations of potential electron acceptors and donors (Jørgensen et al. 1991; Taylor et al. 2001; Zopfi et al. 2001). Instead, the

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discrepancies have been explained by lateral intrusions of additional oxygen (Taylor et al. 2001) or by sufficient  $\text{CO}_2$  fixation by heterotrophic prokaryotes to replenish intermediate substrate pools (anaplerotic metabolism) (Jørgensen et al. 1991; Zopfi et al. 2001). However, the discrepancies are even greater for the high rates of  $\text{CO}_2$  fixation measured below the pelagic chemocline (the water layer where hydrogen sulfide first appears), due to the absence of oxygen and oxidized nitrogen compounds as electron acceptors (Jørgensen et al. 1991; Taylor et al. 2001; Zopfi et al. 2001).

Data concerning the concentration or growth rates of chemolithoautotrophs in pelagic redoxclines are rare. Moreover, usually only specific chemolithoautotrophic groups have been investigated. For instance, nitrifying bacteria have been enumerated either by most probable number (MPN) or by immunofluorescence and fluorescence in situ hybridization detection. The data indicate that these cells account for less than 1% of the total prokaryotes (Ward 1984). An exceptionally high abundance of nitrifiers, up to nearly 8% of total prokaryotes, was reported by Lam et al. (2004) for a deep-sea hydrothermal plume, representing a quite different environment. Casamayor et al. (2001) estimated in estuarine oxic–anoxic interfaces that only 1–2% of the bacteria belonged to the chemolithoautotrophic *Thiobacillus*-like bacteria, although the authors suggested that the use of the MPN technique resulted in an underestimation in the range of at least one order of magnitude. Wirsen et al. (1986) used a culture-dependent method and found that, in an extreme environment (warm vents), facultative and obligate chemolithoautotrophic sulfur-oxidizing bacteria contributed up to 79% of total microbial cells. All of these attempts were based on the detection of chemolithoautotrophic bacteria that gained their energy from a known specific process supported by the respective culture medium.

In contrast, our approach to enumerate chemolithoautotrophic cells is based on flow cytometry, which is a fast and reliable technique for estimating prokaryotic abundance and for distinguishing between different groups of bacterioplankton, based, for example, on their cellular nucleic acid content (Robertson and Button 1989; Li et al. 1995; Lebaron et al. 2002). When used together with radiolabeling and cell sorting, nucleic acid content has been shown to be positively related to cellular activity (Bernard et al. 2000; Lebaron et al. 2001; Servais et al. 2003). Moreover, in combination with molecular analyses of sorted cells, specific activities could be related to different phylogenetic taxa (Zubkov et al. 2001), thereby linking the structures and functions of the bacterial assemblages. Heterotrophic prokaryotes have so far been labeled and sorted using  $^3\text{H}$ - or  $^{35}\text{S}$ -labeled amino acids, whereas  $^{14}\text{C}$ -labeling and cell sorting has been applied only to ultraphytoplankton (Li 1994). In the present study, we adopted the latter approach to investigate the abundance and distinction of chemolithoautotrophic prokaryotes in pelagic redoxclines of the Baltic Sea in order to detect these organisms independently from their specific physiological pathways. The results indicate that this approach is a promising tool in investigations of biotopes with

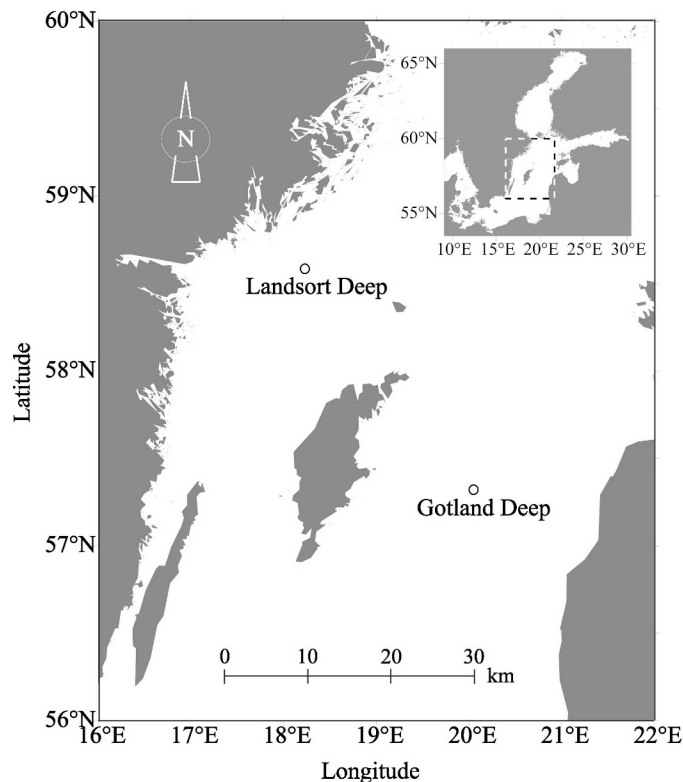


Fig. 1. Map of the central Baltic Sea (courtesy of Jan Donath, Leibnitz-Institut für Ostseeforschung Warnemünde). Circles indicate stations at which dark  $\text{CO}_2$  fixation by bacterioplankton was determined. The inset at the upper right corner shows the entire Baltic Sea.

relatively abundant and actively growing chemolithoautotrophic cells.

## Materials and methods

**Sampling**—Sampling was carried out in February 2006 onboard the RV *Maria S. Merian* during cruise MSM01/1 in the Gotland Basin (Gotland Deep, 57°19.89'N, 20°10.28'E; Landsort Deep, 58°35.00'N, 18°14.06'E) (Fig. 1) in the central Baltic Sea. Water samples were collected in 10-liter or 5-liter Free-Flow bottles (Hydrobios) attached to a polyvinyl chloride-coated stainless-steel conductivity-temperature-depth (CTD)-rosette and were processed immediately.

**Physicochemical structure of the water column**—The physical structure of the water column was recorded by a CTD unit (Seabird 911 plus) equipped with a SBE oxygen sensor (Seabird Electronics). Chemical profiles of oxygen, hydrogen sulfide, ammonia, and nitrate were determined from discrete water samples according to standard methods (Grasshoff et al. 1983).

**In situ  $\text{CO}_2$  dark fixation**— $\text{CO}_2$  dark fixation rates were determined from depth profiles throughout the redoxcline. An anoxic sodium  $^{14}\text{C}$ -bicarbonate stock solution (9.25 MBq  $\text{mL}^{-1}$ ) was prepared by dissolving weighted

sodium  $^{14}\text{C}$ -bicarbonate crystals (SA 1.96 GBq  $\text{mmol}^{-1}$ , Hartmann Analytic GmbH) in deionized water (18 m $\Omega$ ) in serum vials under argon atmosphere in an anoxic chamber. The samples were incubated in 120-mL oxygen bottles that had been filled with an overflow of at least three flask volumes. Immediately before the bottles were closed, 40–60  $\mu\text{L}$  of an anoxic  $^{14}\text{C}$ -bicarbonate stock solution were added with a gas-tight Hamilton syringe. Samples were incubated at in situ temperature ( $\pm 1^\circ\text{C}$ ) in the dark. Incubation was stopped by filtration. To quantify the amount of added  $^{14}\text{C}$ -bicarbonate, 50- $\mu\text{L}$  subsamples were withdrawn from all incubations and placed into scintillation vials containing 50  $\mu\text{L}$  of ethanolamine. The samples were then filtered onto membrane filters (0.2- $\mu\text{m}$  pore size; Whatman, via VWR International), exposed to HCl fumes for 30 min, mixed with scintillation cocktail (UltimaGold XR, Packard), and counted in a TriCarb 2800TR liquid scintillation counter (PerkinElmer).  $\text{CO}_2$  uptake was calculated from the ratio of the  $^{14}\text{C}$ -bicarbonate incorporated and the total inorganic carbon concentration, as determined by the coulometric Single-Operator Multi-Metabolic Analyzer system (Johnson et al. 1993).  $\text{CO}_2$  fixation in control samples, which had been immediately fixed with 1 mL of formalin (37 %) before the addition of  $^{14}\text{C}$ -bicarbonate, was  $0.0062 \mu\text{mol C L}^{-1} \text{d}^{-1}$  (standard deviation  $0.0049 \mu\text{mol C L}^{-1} \text{d}^{-1}$ ). This represented 8–22% of the lowest  $\text{CO}_2$  fixation values from several profiles. All incubations were started within 15 min of sample collection and lasted 23–24 h.

To determine specific fixation rates by distinct prokaryotic clusters, three to four samples collected from discrete depths within the broad layer of enhanced  $\text{CO}_2$  fixation were selected for flow cytometric cell sorting of  $^{14}\text{C}$ -bicarbonate-incubated samples. The prokaryotes were labeled in 12-mL glass vials that had been sealed with a gas-tight injection septum (Exetainer, Labco) and previously flushed with argon for 15 min. Five-milliliter aliquots of the samples were transferred by a syringe into the vials. A stock solution of anoxic sodium  $^{14}\text{C}$ -bicarbonate (500  $\mu\text{L}$ ) was added to the samples, which were then incubated as described previously. Added radioactivity was determined from a 10- $\mu\text{L}$  subsample as described for  $^{14}\text{C}$ -bicarbonate, and another subsample was taken for flow cytometric analysis.

*Flow cytometry of prokaryotes*—Flow cytometric analyses were done on board using a standard FACSCalibur flow cytometer equipped with a cell sorter (Becton & Dickinson). Samples were preserved with paraformaldehyde:glutaraldehyde (1%:0.05% final concentrations), stained with SYBR Green I DNA stain (Marie et al. 1997), and prokaryote abundance was determined. The analyses were routinely done at a flow rate of approximately  $15 \mu\text{L min}^{-1}$  for 3 min and were calibrated by adding 10  $\mu\text{L}$  of a standard bead concentration of  $1.6 \times 10^6$  beads  $\text{mL}^{-1}$  to 333  $\mu\text{L}$  of stained sample. Yellow-green 0.92- $\mu\text{m}$  latex beads (Polysciences) were used as an internal standard. Total bacterioplankton and prokaryotic clusters were differentiated by their flow cytometric signatures on scatter dot plots of  $90^\circ$  light side scatter (SSC) versus green fluorescence (FL1).

Samples incubated with  $^{14}\text{C}$ -bicarbonate were treated as already described and then sorted. From each selected prokaryotic cluster, three subsamples of proportionally increasing numbers of prokaryotic cells (with a range of 1, 2, and  $3 \times 10^3$  to 10, 20, and  $30 \times 10^3$  cells, depending on sample concentration) were flow-sorted, collected on polycarbonate filters (0.2- $\mu\text{m}$  pore size), exposed to HCl fumes for 30 min, and then placed in 20-mL scintillation vials. These samples were mixed with scintillation cocktail (UltimaGold XR, Packard) and counted on board.

The mean  $\text{CO}_2$  uptake per cell of a selected cluster and of all prokaryotes as well as the errors of the measurements were determined as the slope of the linear regression and its standard error of radioactivity versus the number of sorted cells for each of the three subsamples. In addition, total dark  $\text{CO}_2$  fixation was measured by filtering a set of subsamples (e.g., 0.1, 0.2, 0.3 mL) and then calculating volume-specific  $\text{CO}_2$  fixation as the slope of the linear regression of measured radioactivity versus the volume of the filtered subsamples. Sorting purity was assessed routinely by sorting one type of calibration bead with diameters of 0.92  $\mu\text{m}$  from a mixture of two types of beads with different yellow-green fluorescence followed by flow cytometric reanalysis of the sorted material. The sorted material was 99% enriched with the target beads. Three to ten repeated counts of the same sample were made to determine the mean and standard error of prokaryotic cell numbers in a specific cluster used for flow sorting.

## Results

*Profiles of physicochemical parameters, prokaryotes, and dark  $\text{CO}_2$  fixation*—The upper water body was relatively well-mixed by the winter circulation down to the halocline, as indicated by the salinity profile (Fig. 2). A pronounced strong halocline was present at around 80-m depth in the Gotland Deep (Fig. 2a) and at around 70-m depth in the Landsort Deep (Fig. 2c). A distinct maximum in the nitrate profile around the halocline was only found in the Gotland Deep. In both basins, the chemocline was located at a depth of about 115 m. Gradients of hydrogen sulfide and of ammonia were more strongly developed in the Gotland Deep (Fig. 2b). Bottom-water concentrations of hydrogen sulfide and ammonia reached about 40 and 8  $\mu\text{mol L}^{-1}$  in the Gotland Deep and about 20 and 4  $\mu\text{mol L}^{-1}$  in the Landsort Deep, respectively.

Except for slightly enhanced values around the chemocline, prokaryote cell numbers were relatively constant in the assessed depth profiles (Fig. 3). The concentration of total prokaryotes ranged between  $1.16$  and  $1.65 \times 10^6 \text{ mL}^{-1}$  (mean  $1.41 \times 10^6 \text{ mL}^{-1}$ , standard deviation  $0.1 \times 10^6 \text{ mL}^{-1}$ ). Dark  $\text{CO}_2$  fixation increased significantly below the chemocline at both stations. Maximum values of  $1.47 \mu\text{mol C L}^{-1} \text{d}^{-1}$  (Gotland Basin) and  $0.6 \mu\text{mol C L}^{-1} \text{d}^{-1}$  (Landsort Basin) were reached at about 20 m below the chemocline, in the sulfidic zone, at both stations (Fig. 3).

*Flow cytometric differentiation of cell clusters and their contribution to dark  $\text{CO}_2$  fixation*—Flow cytometric scatter plots of  $90^\circ$  light side scatter versus green fluorescence of

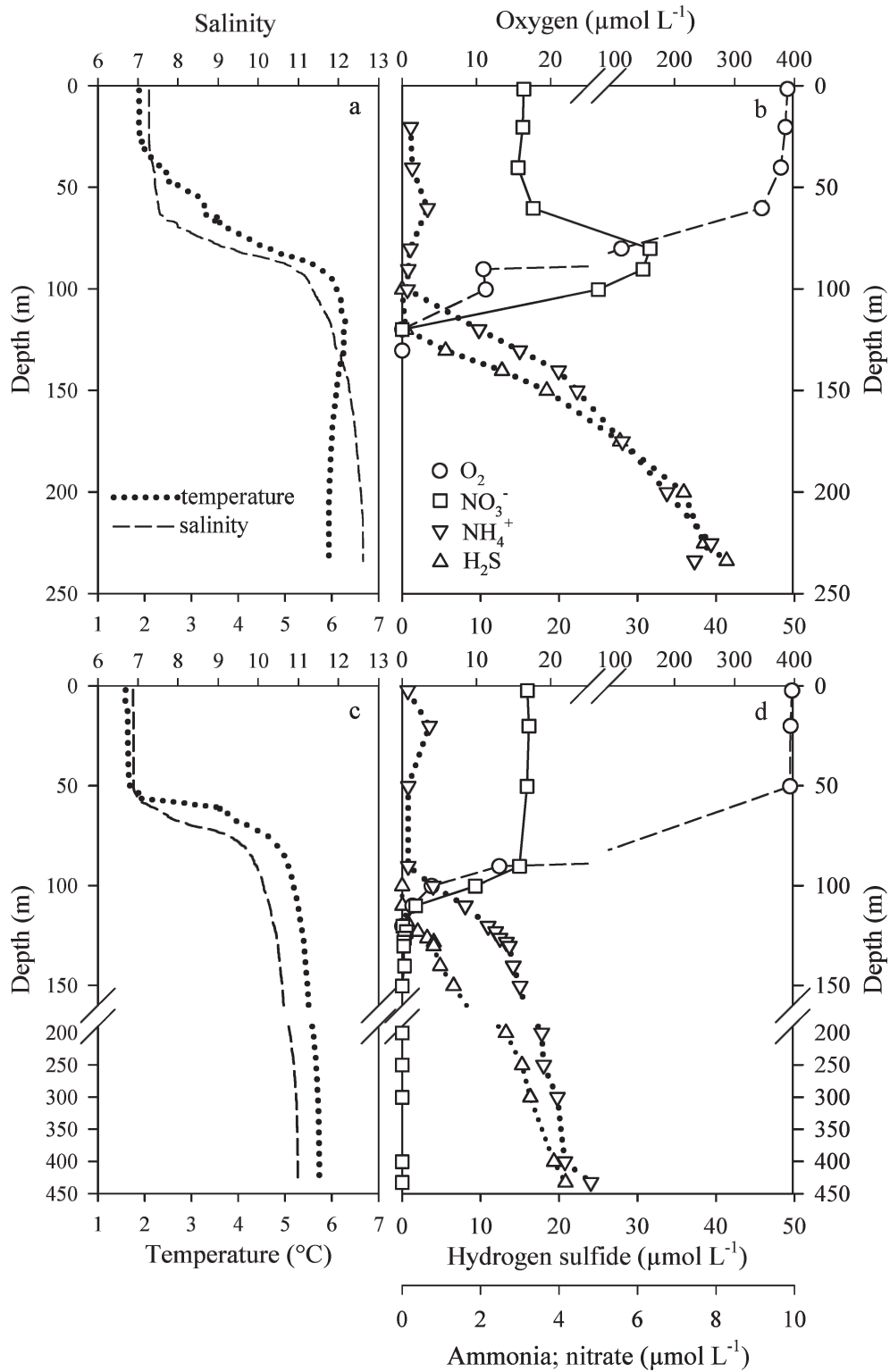


Fig. 2. (a,c) Depth profiles of temperature and salinity and (b,d) oxygen (titrated), nitrate, ammonium, and sulfide of the water column at Gotland Deep (19 February 2006) (a,b) and Landsort Deep (25 February 2006) (c,d).

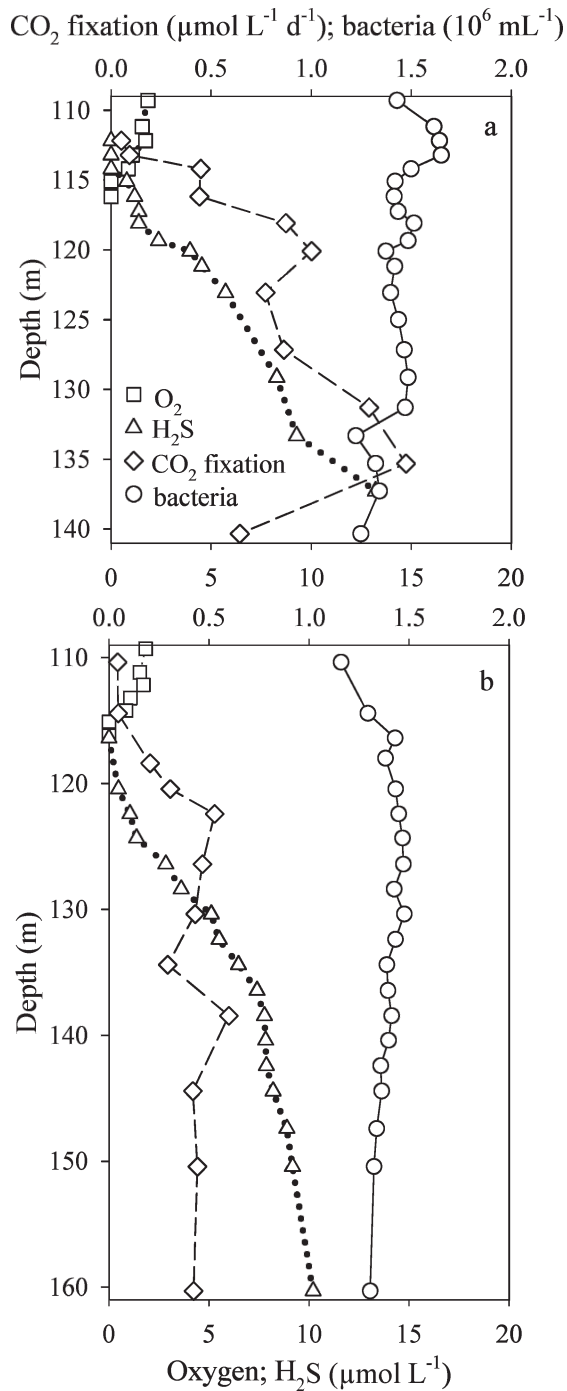


Fig. 3. Sections of the depth profiles showing oxygen, hydrogen sulfide, dark CO<sub>2</sub> fixation, and prokaryotic cell numbers between (a) 110- and 140-m depth at Gotland Deep and (b) 110- and 160-m depth at Landsort Deep.

cells stained with SYBR Green I differentiated five prokaryotic clusters in water samples collected at depths below the chemocline (Fig. 4). The intensity of SYBR Green I staining was interpreted as reflecting the amount of cellular nucleic acids (NA) and permitted differentiation between clusters containing low (LNA) and high (HNA) concentrations per cell. The different clusters were further

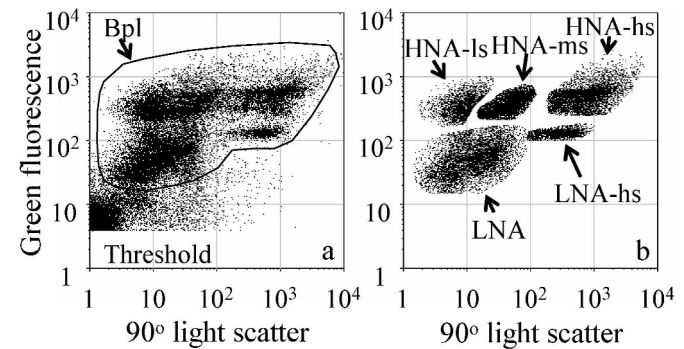


Fig. 4. Characteristic flow-cytometric signatures according to SYBR Green I nucleic acid staining for (a) all bacterioplankton (Bpl) and (b) prokaryotic groups flow-sorted for measurement of cellular dark CO<sub>2</sub> fixation rates. Sample was collected at a depth of 135 m in Landsort Deep. The flow-sorted groups were: Bpl, prokaryotes with low nucleic acid content (LNA); LNA, cells with high side scatter (LNA-hs); HNA, prokaryotes with high nucleic acid content (HNA) and high (HNA-hs), medium (HNA-ms), and low (HNA-ls) side scatter. Arrows indicate the clusters of sorted cells. Solid-line polygon indicates the cluster of bacterioplankton flow-sorted to determine dark CO<sub>2</sub> fixation by a mean Bpl cell.

differentiated into low (ls), median (ms), and high (hs) side scatter (Fig. 4). Clusters HNA-hs and LNA-hs appeared only with the onset of the sulfidic zone and were absent in suboxic and oxic waters (data not shown). The abundance of HNA-hs cells increased while the abundance of HNA-ms cells decreased with depth (Table 1). At concentrations of  $0.25 \times 10^6$  and  $0.43 \times 10^6$  cells mL<sup>-1</sup>, the abundance of HNA-ls and LNA cells, respectively, remained similar in the two basins.

The regression line between total carbon dioxide fixation in the filtered samples and fixation by sorted bacterioplankton cells was close to the 1 : 1 line (Fig. 5). Within the clusters differentiated by flow cytometry, only two, HNA-ms and HNA-hs, showed enhanced CO<sub>2</sub> fixation per cell. This was illustrated by the proportional relationship between the number of sorted cells and <sup>14</sup>C uptake for the respective clusters from two depths of the Gotland Deep (Fig. 6). In addition, <sup>14</sup>C incorporation per cell was always greater in the two active clusters than in total bacterioplankton. Within the clusters LNA and HNA-ls, <sup>14</sup>C incorporation was not detected by this method (Fig. 6a,b). The reproducibility of replicated measurements for the clusters HNA-ms (Fig. 6b) and HNA-hs (Fig. 6c) at 133 m was good. The CO<sub>2</sub> fixation by cells from cluster HNA-hs was much higher, at 150 m compared to 133 m (Fig. 6c), whereas the opposite was true for cells from the HNA-ms cluster (Fig. 6a,b).

At both stations, clusters HNA-hs and HNA-ms represented a relatively stable part of the entire prokaryotic community within the zone of enhanced CO<sub>2</sub> fixation below the chemocline and together accounted for 21–40% of total prokaryotes (Table 1). Based on the estimated specific activity in the different clusters, their relative abundances, and CO<sub>2</sub> fixation by all prokaryotes, we calculated the contribution of the different clusters to overall prokaryotic CO<sub>2</sub> fixation (Table 2). The two active

Table 1. Abundance of all bacterioplankton cells (Bpl) and selected clusters in samples collected at depths below the chemocline ( $\text{H}_2\text{S}$  concentrations given) in Gotland Deep and Landsort Deep, distinguished by flow cytometric sorting. For abbreviations of different clusters, see either the text or the legend of Fig. 4.

Station	Depth (m)	$\text{H}_2\text{S}$ ( $\mu\text{mol L}^{-1}$ )	Abundance (absolute and relative) ( $10^6$ cells $\text{mL}^{-1}$ ) (% of Bpl)		
			Bpl $\pm$ SE	HNA-hs $\pm$ SE	HNA-ms $\pm$ SE
Gotland Deep	124	0.4	1.200 $\pm$ 0.017	0.030 $\pm$ 0.005 (2.5)	0.383 $\pm$ 0.001 (31.9)
	133	7.1	1.310 $\pm$ 0.038	0.033 $\pm$ 0.005 (2.5)	0.486 $\pm$ 0.002 (37.1)
	150	15.5	1.120 $\pm$ 0.020	0.056 $\pm$ 0.003 (5.0)	0.180 $\pm$ 0.008 (16.1)
Landsort Deep	116	0.7	1.410 $\pm$ 0.047	0.020 $\pm$ 0.001 (1.4)	0.396 $\pm$ 0.004 (28.1)
	125	3.7	1.590 $\pm$ 0.022	0.086 $\pm$ 0.006 (5.4)	0.406 $\pm$ 0.006 (25.5)
	130	5.4	1.520 $\pm$ 0.060	0.201 $\pm$ 0.008 (13.2)	0.240 $\pm$ 0.004 (15.8)
	135	6.1	1.430 $\pm$ 0.017	0.211 $\pm$ 0.021 (14.8)	0.221 $\pm$ 0.017 (15.5)

SE, standard error of three to four measurements.

clusters HNA-hs and HNA-ms together contributed between 66% and 100% of the measured prokaryotic  $\text{CO}_2$  fixation. In the upper part of the anoxic zone,  $\text{CO}_2$  fixation was always dominated by the HNA-ms cluster, with respect not only to its total contribution but also to its cell-specific activity (Table 2). Cluster HNA-hs did not contribute significantly to  $\text{CO}_2$  fixation just below the chemocline (124 m). In contrast, its contribution to total  $\text{CO}_2$  dark fixation in deeper layers accounted for as much as (Gotland Deep) or even more than (Landsort Deep) that of the HNA-ms cluster. This was evidenced by the total contribution and cell-specific fixation rates of the HNA-hs cluster (Table 2). Cell-specific  $\text{CO}_2$  fixation in the two active clusters in the two basins ranged from 3.5 to 24.7 fg C

$\text{cell}^{-1} \text{d}^{-1}$ . The cells of other clusters never reached comparable values; their activities, computed as the difference between the activity of total bacterioplankton and the sum of the activities of the two other clusters, were 0.1 and 0.9 fg C  $\text{cell}^{-1} \text{d}^{-1}$ , with only one exception of 2.2 fg C  $\text{cell}^{-1} \text{d}^{-1}$  (Table 2).

## Discussion

In the present study, dark  $\text{CO}_2$  fixation measurements and flow cytometric cell sorting of samples originating around the maximum  $\text{CO}_2$  fixation zone, i.e., in sulfidic waters about 20 m below the chemocline, together provided evidence for the existence of an abundant population of chemolithoautotrophic cells, representing about 30% of total prokaryotes. These cells belonged to two clusters and were responsible for 65–100% of the total dark fixation determined. Calculated cell-specific carbon fixation rates were in the range of 3.5 to 24.7 fg C  $\text{cell}^{-1} \text{d}^{-1}$ . According to our knowledge, this is the highest abundance of chemolithoautotrophic prokaryotes reported for a pelagic marine environment. It is also the first attempt to estimate the number of chemolithoautotrophic prokaryotes by combining  $^{14}\text{CO}_2$  uptake with cell sorting and activity measurements.

The measured values of dark  $\text{CO}_2$  fixation were in the same range as previously reported values, not only from these stations (Labrenz et al. 2005) but also from other marine redoxclines, such as the Black Sea (Jørgensen et al. 1991) and the Cariaco Trench (Taylor et al. 2001). In all of these studies, maximum activity was found in the anoxic layer and clearly below the chemocline.

The amount of total  $\text{CO}_2$  fixation measured in a discrete volume of filtered sample corresponded well with that measured from sorting all cells from the same volume. The results indicated that only suspended bacterioplankton were responsible for the dark  $\text{CO}_2$  fixation measured in the sample (Fig. 5). Thus, the contribution, if any, by aggregated prokaryotes was negligibly small; otherwise, there would have been a significant difference between these values because aggregates cannot be assessed by flow cytometry. The coincidence between the appearance of a prokaryotic cluster with high side scatter and the significant increase in dark  $\text{CO}_2$  fixation just below the

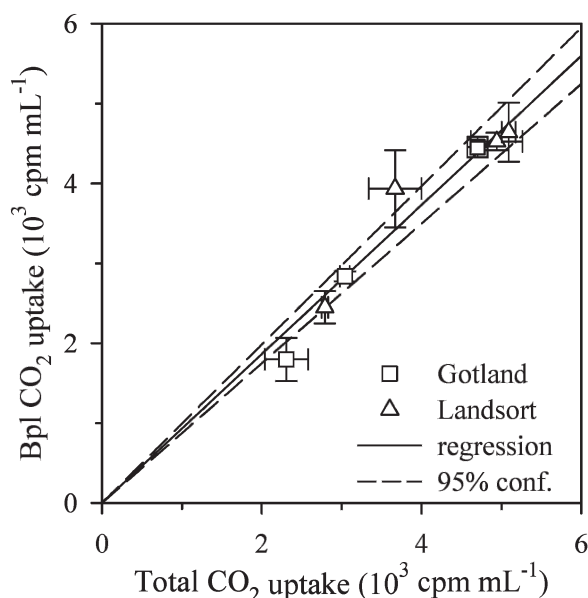


Fig. 5. Comparison of total  $^{14}\text{CO}_2$  fixation from filtered samples vs.  $^{14}\text{CO}_2$  fixation in flow-sorted prokaryotes (Bpl) in the same water samples (for explanations, see text). Activity is given in counts per minute (cpm). Samples were collected in Gotland Deep and Landsort Deep. Error bars indicate a single standard error of triple measurements. Solid line shows a linear regression, and dashed lines indicate 95% confidence intervals. The corresponding regression slope is  $0.93 \pm 0.03$ , regression coefficient is 0.95, and probability value  $P < 0.0001$ .

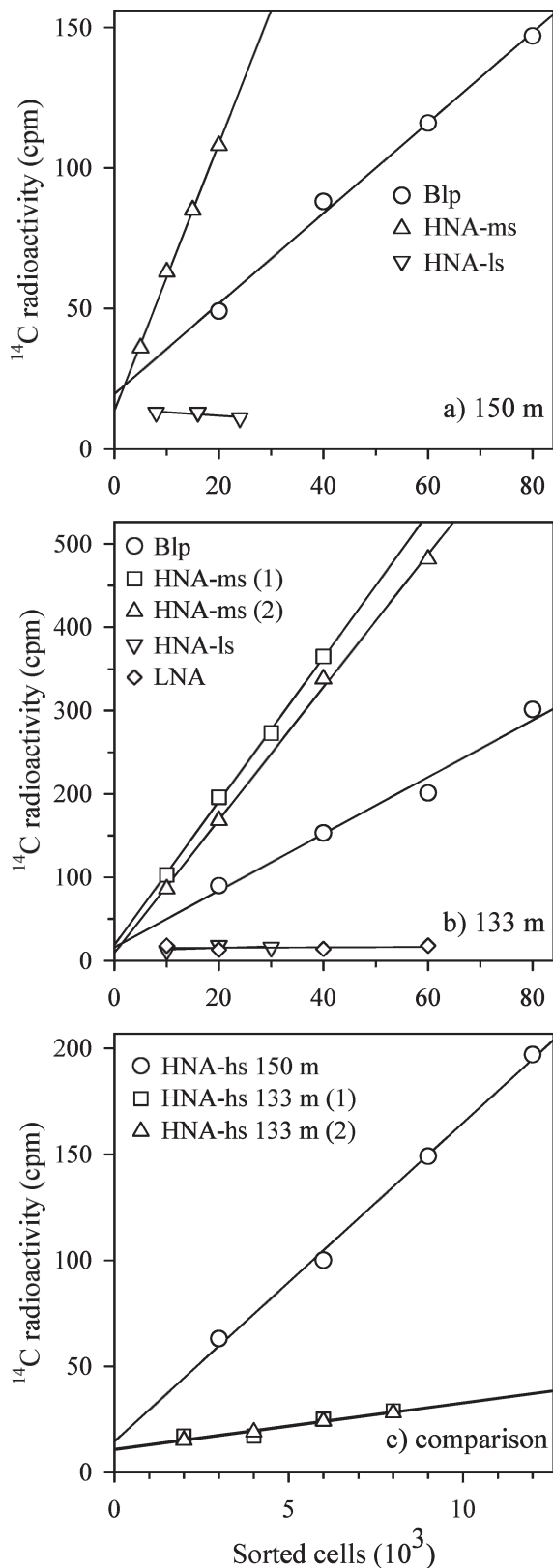


Fig. 6. Flow-cytometric sorting radioassays of  $^{14}\text{CO}_2$  fixation, determined by flow cytometrically sorted average bacterioplankton (Bpl), HNA-ms, and HNA-ls cells from Gotland Deep at (a) 150-m and (b) 133-m depth. (c) Comparison of HNA-hs

chemocline suggested that the cells of this cluster were primarily responsible for the increased fixation of  $^{14}\text{CO}_2$ . The clear separation of cluster HNA-hs from the other prokaryotic clusters (Fig. 4) and its sufficient abundance allowed the application of flow cytometric sorting and measurements of  $^{14}\text{CO}_2$  incorporation (Fig. 6).

The calculated values of specific carbon fixation by clusters HNA-ms and HNA-hs, shown in Table 2, were minimum estimates, as heterotrophic prokaryotes also might have been present in these clusters. However, because the specific fixation rates exceeded  $10 \text{ fg C cell}^{-1} \text{ d}^{-1}$ , most cells of the cluster can be considered as chemolithoautotrophic prokaryotes. At a mean carbon content of about  $20 \text{ fg C cell}^{-1}$  (Lee and Fuhrman 1987), these rates imply generation times of less than 48 h, which are at the upper end of the growth rates that can be realized in this environment. Weinbauer et al. (2003) reported production rates for heterotrophic prokaryotes from the Gotland Deep. These authors measured generation times of more than 48 h in suboxic water depths and significantly above 100 h in anoxic depths. We assessed heterotrophic  $\text{CO}_2$  fixation by anaerobic reactions, using 8% as the upper end of the reported values (Li 1982; Roslev et al. 2004). At a generation time of 48 h, this resulted in a specific  $\text{CO}_2$  fixation rate of about  $0.8 \text{ fg C cell}^{-1} \text{ d}^{-1}$ . Since bacterial growth rates are thought to decrease in anoxic water layers (Weinbauer et al. 2003), the specific  $\text{CO}_2$  fixation rates of heterotrophic cells should be even lower and thus well in agreement with the activity of the remaining cells outside clusters HNA-ms and HNA-hs. Therefore, the large differences in cell-specific  $\text{CO}_2$  fixation between these two clusters and the others underscore the importance of chemolithoautotrophic prokaryotes in  $\text{CO}_2$  fixation relative to heterotrophic bacteria.

For most of the measured  $\text{CO}_2$  fixation, oxygen as well as nitrate and nitrite (not shown) could be ruled out as electron acceptors for chemolithoautotrophic prokaryotes because of their decreasing gradients, down to undetectable levels, at the chemocline (Fig. 2). The unavailability of these electron acceptors at zones of maximum  $\text{CO}_2$  fixation raises the question of what drives this reaction. One possibility is the disproportionation of sulfur compounds, which depends on the scavenging of sulfide by oxidized iron or manganese (Thamdrup et al. 1993). There is no proof as yet for the direct involvement of manganese oxide as an electron acceptor for chemolithoautotrophic sulfur oxidation; however, it seems to be of limited importance due to the relatively low amounts of oxidized manganese produced (Neretin et al. 2003) and the low energy yield of disproportionation. The problem of available electron acceptors and donors that explain the measured values of  $\text{CO}_2$  fixation has been discussed extensively (Jørgensen et

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$^{14}\text{CO}_2$  fixation at the two depths. Activity is given in counts per minute (cpm). Numbers in parentheses indicate replicated sortings of the same sample completed on consecutive days. Solid lines show linear regressions.

Table 2. Dark CO<sub>2</sub> fixation of all prokaryotic cells (Bpl) and of the major chemoautotrophic clusters. Cell-specific dark CO<sub>2</sub> fixation was calculated using the abundances given in Table 1 for these two populations at depths below the chemocline (H<sub>2</sub>S concentrations given) in Gotland Deep and Landsort Deep. Cell-specific fixation of the remaining bacterioplankton cells (Other) was calculated from the respective differences. For abbreviations of different clusters, see either the text or the legend of Fig. 4.

Station	Depth (m)	H <sub>2</sub> S (μmol L <sup>-1</sup> )	Population CO <sub>2</sub> fixation (μmol C L <sup>-1</sup> d <sup>-1</sup> )				Cell-specific CO <sub>2</sub> fixation (fg C cell <sup>-1</sup> d <sup>-1</sup> )		
			Bpl±SE	Sum	HNA-hs±SE	HNA-ms±SE	HNA-hs	HNA-ms	Other
Gotland Deep	124	0.4	0.36±0.01	0.33 (92%)	*	0.332±0.039	*	10.4	0.50
	133	7.1	1.06±0.12	1.02 (96%)	0.017±0.003	1.000±0.030	6.2	24.7	0.68
	150	15.5	0.11±0.01	0.10 (91%)	0.051±0.005	0.052±0.004	10.9	3.5	0.08
Landsort Deep	116	0.7	0.29±0.02	0.22 (76%)	n.d.	0.216±0.015	n.d.	6.6	0.89
	125	3.7	0.58±0.07	0.38 (66%)	n.d.	0.379±0.015	n.d.	11.2	2.2
	130	5.4	0.35±0.01	0.31 (89%)	0.219±0.022	0.096±0.013	13.1	4.8	0.37
	135	6.1	0.38±0.03	0.40 (104%)	0.316±0.046	0.081±0.011	18.0	4.4	n.c.

Bpl, estimated from cell sorting. Sum, sum of carbon fixation by the HNA-hs and HNA-ms populations, where percentages of total prokaryotic fixation are given in parentheses. SE, standard error of three to four measurements; n.d., not determined because of either low cell abundance or low cellular radioactivity; n.c., not calculated.

\* Values statistically not significant different from zero.

al. 1991; Taylor et al. 2001; Zopfi et al. 2001). Jørgensen et al. (1991) and Zopfi et al. (2001) proposed that the discrepancy could be explained by the contribution of heterotrophic prokaryotes, via anaplerotic fixation of CO<sub>2</sub>. In contrast, Taylor et al. (2001) stressed the importance of chemolithoautotrophic prokaryotes in the measured carbon fixation rates and calculated that the activity of heterotrophic bacteria could not contribute more than 2% to the reaction. This conclusion is supported by our results from the pelagic redoxcline of the Baltic Sea.

Since cell-specific CO<sub>2</sub> fixation within clusters HNA-ms and HNA-hs was much higher than that explainable by anaplerotic CO<sub>2</sub> fixation (<0.8 fg C cell<sup>-1</sup> d<sup>-1</sup>), these clusters from the relevant depth layers should have been dominated by chemolithoautotrophic prokaryotes. Accordingly, these clusters were assumed to consist of about 240,000–520,000 cells mL<sup>-1</sup> (Table 1). Specific fixation rates of 3.5–6.6 fg C cell<sup>-1</sup> d<sup>-1</sup> (Table 2) within clusters HNA-ms and HNA-hs likely indicated that most of the cells belonged to slowly growing chemolithoautotrophic prokaryotes rather than to an increasing proportion of heterotrophic prokaryotes. Due to their low abundance, cells of cluster HNA-hs from the upper two depths of the Landsort Deep were not sorted. This most likely contributed to the higher mean specific carbon fixation rates in the other clusters (0.8 and 2.2 fg C cell<sup>-1</sup> d<sup>-1</sup> compared with 0.08–0.68 fg C cell<sup>-1</sup> d<sup>-1</sup>; Table 2). Based on the calculation for anaplerotic CO<sub>2</sub> fixation by heterotrophic prokaryotes and mean generation times of more than 48 h, a certain number of chemolithoautotrophic prokaryotes within the other clusters, with mean cell-specific CO<sub>2</sub> fixation between 0.4 and 0.9 fg C cell<sup>-1</sup> d<sup>-1</sup>, would be expected (Table 2). If the cell concentration of the two active clusters is assumed to be the minimum estimation for chemolithoautotrophic cells, then 20–40% of the bacterial assemblages in this zone are chemolithoautotrophic. This is well in agreement with previous estimated concentrations (90,000–560,000 cells mL<sup>-1</sup>), based on enrichment experi-

ments, of chemolithoautotrophic prokaryotes at the chemocline of the Gotland Deep (Labrenz et al. 2005).

The distribution of active chemolithoautotrophic prokaryotes within at least two different clusters and the opposing importance of the two clusters at different depths suggest the presence of different groups of chemolithoautotrophs in this layer of enhanced dark CO<sub>2</sub> fixation. Alternatively, the determined reaction rates may have been due to the same microorganism switching its metabolism with depth, i.e., from oxidizing H<sub>2</sub>S to thiosulfate at lower H<sub>2</sub>S concentrations to oxidizing H<sub>2</sub>S to molecular sulfur at higher H<sub>2</sub>S concentrations. The molecular sulfur deposited inside cells may have been the reason for a higher side scatter, as previously shown for anoxygenic photosynthetic bacteria (Casamayor et al. 2007).

The high numbers of chemolithoautotrophic prokaryotes and their appearance in separate and unique clusters should enable testing of the above-stated hypotheses by cell sorting in combination with molecular analysis of the sorted cells (Zubkov et al. 2001). Potential candidates could belong to the *Epsilonproteobacteria*, since their appearance around the redoxcline coincides with that of cluster HNA-hs (Labrenz et al. 2007). In the Cariaco Basin, Lin et al. (2007) found a similar distribution of *Epsilonproteobacteria* around the redoxcline. From the abundant distribution of these bacteria in this layer and their under-representation as determined by leucine uptake, members of this group should belong to chemolithoautotrophs. Identification of the relevant phylogenetic types of prokaryotes will contribute to an understanding of the mechanisms fueling chemolithoautotrophic CO<sub>2</sub> fixation under anoxic conditions.

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